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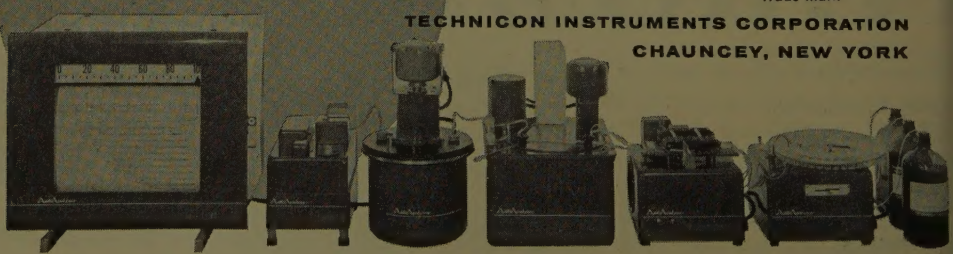

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# ACQUIRED CIRCULATING ANTICOAGULANT IN CLASSICAL HEMOPHILIA

By O. EGEBERG

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(Received for publication February 11, 1961)

A number of reports have been given on patients with hemophilia in which the original clotting factor deficiency has been complicated by the development of circulating anticoagulants. Most of these patients have had hemophilia A (classical hemophilia) with an inhibitor directed against factor VIII (antihemophilic A factor = AHA), and the complication has been diagnosed a shorter or longer time after transfusions with blood, plasma or plasma fractions. Hougie, in 1955, recorded 18 published cases of hemophilia A and B (Christmas disease) with inhibitor against the deficiency factor. A limited number of new cases have since then been published. In addition to the cases of circulating inhibitors in hemophiliacs, similar inhibitors have been described in a small number of non-hemophiliacs; namely in some females in the first months after parturition, and in males or females with different diseases, *e. g.* chronic nephritis, lymphadenopathia, or collagen disease.

An analysis of the effect of the inhibitor on the patient's own blood has usually been hampered by lack of exact knowledge of the patient's original clotting defect before the appearance of the inhibitor. This paper gives the results of a study on a new case of acquired circulating inhibitor in a patient with hemophilia A. The clotting system of the patient was to a certain degree investigated beforehand, and samples of plasma from before and after the development of the inhibitor could be analyzed and compared. In connection with the investigations, a simple assay method of AHA inhibitor activity is presented. The study afforded opportunity for considerations of the question whether patients with severe hemophilia A are completely devoid of AHA protein or not.

## MATERIALS AND METHODS

### *Materials.*

*Calcium.* Optimal concentrations of calcium chloride were used in all clotting tests. The solutions were made by diluting a 1 M aqueous stock solution in distilled water.

*Cephalin suspension.* A stock cephalin suspension was prepared according to the method of Hjort, Rapaport & Owren (1955). The suspension was diluted in Owren's bufer (Owren 1947) to optimal concentration in the cephalin assays, and stored, as the stock suspension, at —20° C. It was thawed at 37° C, and shaken vigorously before use.

*Citrated "silicone" plasma.* Venepunctures were done with siliconized needles. The first 2—3 ml of blood were discarded. Nine parts of blood were collected into chilled siliconized tubes containing one part of 3.1 g per cent sodium citrate dihydrate. After mixing, the blood was centrifuged at 2,500 rpm. (max. *g* ca. 1,800) for 30 min. at 4° C. The plasma was pipetted off with a siliconized pipette, and stored at —20° C in small aliquots in siliconized tubes. Thawing of plasma was done in water bath at 37° C, and it was kept in ice bath until use in the assays. This technique was applied both for preparation of the different test plasmas and the special factor-deficient plasmas used as substrates in the assays.

*Oxalated "silicone" plasma.* This was only used when plasma was adsorbed with barium sulphate. Blood was collected into tubes containing 1/10 volume of 2.5 g per cent potassium oxalate monohydrate. Plasma preparation and storage were as for citrated plasma.

*Dilution fluids.* For dilutions of plasma, two different fluids were applied, I and II (Hjort, Rapaport & Owren 1955). The plasma was first diluted in citrated saline (fluid I) to 10 times the final concentration, then the final dilution 1/10 was done in buffered citrated saline (fluid II).

*Serum.* Blood was collected in siliconized glass tubes, kept at 37° C for three hours, and centrifugated at 2,500 rpm. for 30 min. at room temperature. Serum was collected and kept in siliconized glass tubes in water bath at 37° C for 24 hours. After re-centrifugation, the serum was separated from the sediment and stored in small aliquots at —20° C in siliconized glass tubes. Thawing w done just before use, as for "silicone" plasma.

*Siliconizing of equipment.* Blood, plasma a plasma dilutions were handled with strict silico technique, except for the glass test tubes, whi were unsiliconized.

### *Methods.*

*Adsorption.* Citrated plasma was adsorbed siliconized glass tubes with 1/10 volume of 25 p cent (V/V) aqueous solution of aluminum hydro ide from a standard gel from Cutter Laboratori Berkeley, California, U.S.A. The mixture w shaken and kept at 37° C for 3 min. Centrifugati was done at 2,500 rpm. for 20 min. at 4° C, plasma separated from sediment and stored at —2 C. Oxalated plasma was adsorbed with 100 mg barium sulphate per ml. Adsorbtion was done room temperature in a siliconized glass tube wh was tilted for 5 min. After centrifugation the pl ma was separated and stored as described. Thaw and handling of adsorbed plasma were as for dinary citrated plasma.

*Dialysis* of plasma was done in cellophe casings from Viscing Corporations, Chicago, U.S The plasma was dialyzed for 20 hours at 5° against four changes of a large volume of dilut fluid I.

*Assaying of AHA, AHB (antihemophilic factor = Christmas factor = factor IX), and A (antihemophilic C factor = plasma thrombopla antecedent = PTA)* was done in one-stage ceph systems, using plasmas from patients with sev deficiencies of the respective factors as substra The methods have been described in detail e where (Egeberg 1961).

*Assay of AHA inhibitor.*

*Principle:* The residual AHA level was timated in a normal human plasma (indic plasma) after a standard incubation with dil inhibitor plasma for a specified time. As b control was used the residual AHA level in same indicator plasma after corresponding inc tion with dilution fluid only.

The following definition of one unit of inhib activity was used: Diluted inhibitor plasma defined to contain one unit of inhibitor activity ml, when a mixture with the undiluted indic

plasma in the ratio 9/1, after an incubation for one hour at 37° C, showed a reduction of AHA to 25 per cent of the control value.

This unit definition and the incubation systems are similar to those of Biggs & Bidwell (1959). They worked, however, with solutions of pig AHA concentrates as indicator material, containing about 10 times the AHA activity of normal human plasma. Further, they used incubation of the indicator material with diluted ordinary hemophilia A plasma as control, and the AHA values were measured in an assay based on the thromboplastin generation test.

*Technical procedure.* Indicator plasma was prepared from a healthy adult person with AHA level near to the normal average. 0.1 ml indicator plasma + 0.9 ml dilution fluid II were mixed in a siliconized glass tube, and incubated at 37° C for one hour (reference tube). A second tube, containing 0.1 ml indicator plasma + 0.9 ml diluted inhibitor plasma, was incubated in the same way. Immediately following the one hour incubation, the AHA levels were determined in the two samples. An adequate dilution of the inhibitor plasma was determined beforehand by preliminary tests in order to give a reduction of AHA in the indicator plasma to about 25 per cent of the control value of the reference tube (reference 100 per cent).

The deepfrozen reagents for the AHA test system were thawed shortly before the pre-incubation was finished. As assay mixture for AHA, 0.2 ml cephalin suspension + 0.2 ml sample directly from the pre-incubation mixture (indicator plasma diluted 1/10) + 0.2 ml undiluted hemophilia A substrate plasma were pipetted into each of four new glass test tubes, and incubated at 37° C. After 6 min. icubation, the mixtures were recalcified with 0.2 ml 30 mM calcium chloride, pre-warmed to 37° C, and the clotting times determined. The end points were read, after regular tilting of the tubes, when coagulation spread generally in the mixtures. The clotting time was calculated as the average of the four simultaneous runs. AHA values were determined in per cent of normal level from a calibration curve, constructed by testing different dilutions of normal plasma in the same assay system.

From these AHA determinations, the residual AHA value corresponding to the applied dilution of inhibitor plasma was calculated in per cent of the control value. If this residual AHA was found outside the range of 10—40 per cent, the procedure was repeated with a more adequate dilution of the inhibitor plasma. The strength of the inhibitor activity in units was interpolated from a standard dilution curve for inhibitor plasma (see text).

*Other assay methods,* see Table I.

## CASE REPORT

The patient, K. S., a 29-year old male, had a severe hemorrhagic diathesis since early childhood. Two brothers had died from bleeding after small traumas, and there were also other bleeders in his mother's family. Before the age of five he had been hospitalized four times for bleeding and hemarthroses. He then had relatively few troubles until the age of 22, when a retro-peritoneal bleeding caused a new hospitalization, during which he was transfused with 500 ml blood. Shortly afterwards he was re-admitted twice because of bleeding after tooth ectractions, and received altogether 3,500 ml blood and 2,500 ml plasma. At the age of 25, he was given 1,800 ml blood, also because of tooth ectractions. In 1957, his disease was classified as hemophilia of type A, and no AHA could be demonstrated in his plasma. After a transfusion with 750 ml normal plasma, the concentration of AHA in his blood rose to the expected level. One year later, his blood was specially examined for circulating inhibitor against AHA, but none was detected. Since then, his blood was repeatedly under control, and the clotting tests excluded the presence of specific inhibitor.

December 3, 1959, he was admitted to Medical Department A, Rikshospitalet, Oslo, for dental treatment. Six days earlier some blood had been taken from him and plasma prepared and stored in aliquots in standard manner at —20° C.

At admittance he was generally in good condition, but the dental state was bad, with extensive caries and infected teeth, one of which had to be extracted. Because the patient on previous occasions had bled severely after extractions, he was given transfusions pre- and post-operatively, receiving in all 500 ml blood and 1,700 ml plasma. Only moderate bleeding followed the tooth extraction, and the patient could be discharged on December 15.

Table I. *Results of routine tests of patient's clotting system, measured in the last plas batch before (I) and in the first batch after his last transfusions (II).*

| Test | Results of tests | | Normal range | Methods |
|---|---|---|---|---|
| | Plasma I | Plasma II | | |
| Thromboplastin time | 14.6 sec. | 13.9 sec. | 12.5-15 sec. | Quick's system with hum brain thromboplastin |
| Recalcification time | 590 sec. | 615 sec. | 95-130 sec. | Waaler (1957) |
| Cephalin time | 255 sec. | 259 sec. | 57-64 sec. | Egeberg (1961) |
| Fibrinogen | 295 mg/100 ml | 312 mg/100 ml | 150-400 mg/100 ml | Jacobsson (1955) modifi Blombäck & Blombäck(19 |
| Prothrombin | 96 % | 91 % | 75-120 % | Hjort *et al.* (1955) |
| Factor V (proaccelerin) | 113 % | 103 % | 70-150 % | Owren (1947) |
| Factor VII (proconvertin) | 107 % | 115 % | 75-125 % | Owren & Aas (1951) |

On March 1, 1960, a new blood sample was taken from the patient, and when this was examined, the plasma showed reactions which could be explained only by the presence of circulating inhibitors. After that he was repeatedly controlled, and the titer of the inhibitor activity estimated. He had no severe bleeding episodes in the time following the transfusions.

## RESULTS

1. *The diagnosis of inhibitor activity.*

In the following, the plasma prepared prior to patient's last transfusions is referred to as plasma I, and the plasma prepared from blood taken 95 days later, 82 days after the transfusions, is referred to as plasma II.

As shown in Table I, thromboplastin time, recalcification time and cephalin time were practically the same in plasma I and II. The cephalin time of plasma I had been controlled repeatedly, and had not changed significantly during the storage of the plasma. Fibrinogen, prothrombin, factor V (proaccelerin) and factor VII (proconvertin) were found within normal ranges, and roughly at the same levels in the two batches.

No AHA activity could be demonstrated in plasma I and II by the technique used. Measuring of AHB and AHC showed lo values in plasma II than in plasma I w tested in plasma dilution 1/10. With as dilution increased to 1/50, and incubat the assay mixture only for 3 min., the val of AHB and AHC were found normal both plasma I and II, and also for th factors roughly at the same levels in the batches.

Cephalin time of mixtures of patie plasma and normal plasma gave, howe a completely different pattern for plasm and II. A mixture of plasma II and nor plasma in the ratio 9/1 gave a cephalin t of 150 sec. after the ordinary assay incu tion of 6 min.; a corresponding mixture plasma I clotted in 90 sec. The cephalin t for the mixture with plasma II increa rapidly when increasing the incubation t in contrast to the mixture with plasm See Fig. 1.

Fig. 2 illustrates the results of ceph time determinations of mixtures of nor plasma and patient's plasma I and I various proportions. Curve 1 shows plasma I was corrected in the same wa ordinary hemophilic plasma (Curve 3),

a marked correction of the prolonged cephalin time after mixing, *e. g.* with only 10 per cent normal plasma. Curve 2 demonstrates a significantly decreased correcting effect of normal plasma on patient's plasma II. An increase in the incubation time from 6 to 12 min. further accentuated the abnormality (Curve 4).

These results were then in accordance with the presence of an inhibitor directed against AHA, and all other tests showed correspondance with this hypothesis.

2. *Cephalin time of mixtures of patient's inhibitor plasma with his non-inhibitor plasma and with plasma from other hemophiliacs.*

Investigations were made to see if addition of plasma II, with inhibitor activity, could prolong the cephalin time of plasma I. Plasma samples from the two batches were mixed in different proportions, but no significant change in the cephalin time occurred. Neither did addition of small amounts of plasma II to the plasmas from two other patients with ordinary hemophilia A of severe type tend to prolong their cephalin time.

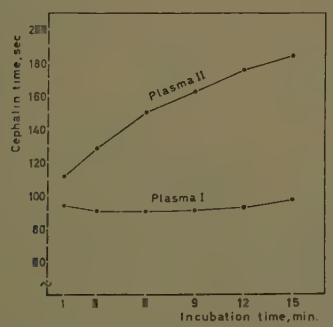


Fig. 1. *Variations in incubation duration in cephalin time determinations of mixtures of patient's plasma and normal plasma in the ratio 9/1.* Incubation of normal plasma with patient's plasma from before (I) and after development of the inhibitor (II).

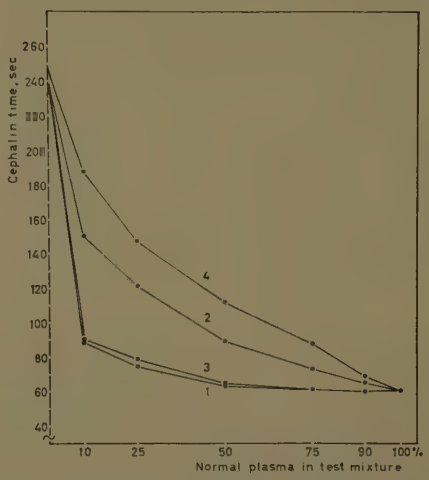


Fig. 2. *Cephalin time of mixtures of normal plasma and hemophilic plasma, with and without inhibitor.* Mixtures of normal plasma and plasma from 1) patient K. S. before transfusions (I), 2) K. S. after transfusions (II), 3) an ordinary patient with severe hemophilia A, 4) K. S. after transfusions (II). Incubation time in cephalin assay prolonged from 6 to 12 min.

3. *Assaying of AHA inhibitor.*

Fig. 3 illustrates the ability of plasma II to inhibit normal AHA. The level of AHA in a normal plasma was determined after pre-incubation with different dilutions of inhibitor plasma, as previously described. Interpolating on the curve fitted to the determined points, it follows that a 1/125 dilution of plasma II destroyed 75 per cent of the AHA content of normal plasma under the standardized conditions. Consequently, the plasma had 125 units of inhibitor activity per ml.

A dilution curve as that shown in Fig. 3, can be used as assay curve for the estimation

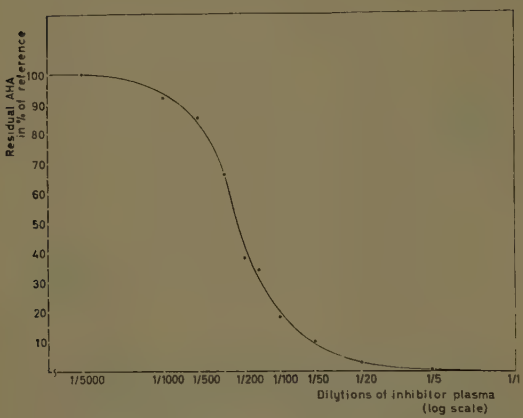


Fig. 3. *Dilution curve for AHA inhibitor activity.* Inhibitor plasma (II) was diluted and then mixed with normad plasma (indicator plasma) in proportion 9/1. The mixture was incubated at 37° C for one hour. Residual AHA was measured in the mixtures with different dilutions of inhibitor plasma, and expressed as per cent of residual AHA after corresponding incubation of the same normal plasma with dilution fluid only (reference value).

of AHA inhibitor activity in other plasmas or in other materials. The plasma to be tested is diluted to give an inhibitor effect corresponding to a residual AHA between 10 and 40 per cent of reference value. The inhibitor concentration of the dilution is then determined by interpolating on the curve, and the total strength in the plasma is computed from the dilution ratio.

4. *The effect of the inhibitor on partially AHA deficient plasma.*

The inhibitor plasma produced a decrease of AHA also when mixed with plasma from ordinary hemophiliacs with a partial AHA deficiency, and with plasma from patients with von Willebrand's disease. The effect was roughly as could be expected from the known effect on normal plasma. See Table II.

Table II. *Results of mixing inhibitor plasma with plasmas from patients with partial deficiency of AHA. The AHA levels were first determined for the partial deficiency plasmas with the ordinary assay method. Mixtures of diluted inhibitor plasma and undiluted partial deficiency plasmas in the ratio $^9/_1$ were then pre-incubated at 37° C for one hour, and the residual AHA in per cent of normal level was determined. Correspondingly, the AHA values were determined after the same incubation of partial deficiency plasmas with dilution fluid only. The diluted inhibitor plasma had an activity of one unit per ml.*

| Test material | Without pre-incubation | After pre-incubation for one hour at 37° C | |
|---|---|---|---|
| | | With diluted inhibitor plasma | With dilution fluid II |
| | AHA % of normal | Residual AHA % of normal | Residual AHA % of normal |
| Plasma from hemophiliacs with partial deficiency of AHA | | | |
| Pat. 1. E. J. | 17 | 3 | 13 |
| Pat. 2. R. N. | 20 | 4 | 15 |
| Plasma from patients with von Willebrand's disease | | | |
| Pat. 3. A. R. | 44 | 6 | 36 |
| Pat. 4. G. H. | 5 | <1 | 4 |

Table III. *Inhibitor activity in patient's citrated plasma prepared from blood taken at different times after his last transfusions.*

| Time between the last transfusion and blood sample Days | Plasma stored at — 20° C Days | Inhibitor activity Units per ml |
|---|---|---|
| 82 | 100 | 125 |
| | 230 | 102 |
| 154 | 27 | 113 |
| 180 | 1 | 78 |
| 287 | 6 | 11 |

5. *Control of the titer of inhibitor in the patient.*

Table III shows that the inhibitor in the blood of patient K.S. slowly decreased in activity as time elapsed after his last transfusion. After 287 days, the remaining activity was less than 1/10 of the activity first measured.

6. *Characteristics of the inhibitor.*

The inhibitor activity decreased only slowly on storage of plasma at —20° C, see Table III.

The inhibitor was not dialyzable. Dialysis of plasma II for 20 hours against dilution fluid I gave a reduction of the activity only from 125 to 112 units per ml.

As seen from Table IV, the inhibitor was not adsorbed with aluminum hydroxide, nor, correspondingly, was it removed by adsorption of patient's oxalated plasma with barium sulphate. Serum also showed high levels of inhibitor activity, see Table IV.

Table IV. *Inhibitor activity in different test materials prepared from patient's blood taken 180 days after his last transfusion.*

| Test material | Inhibitor activity Units per ml |
|---|---|
| Citrated plasma | 78 |
| Citrated plasma adsorbed with $Al(OH)_3$ | 87 |
| Citrated serum | 56 |

Table V. *Results of heating on inhibitor activity. Inhibitor plasma was dituted $^1/_{10}$ in dilution fluid II and heated for 10 min. After centrifugation at 2,500 rpm. for 30 min. the upper layer of the diluted plasma was collected and stored at –20° C until assaying. Inhibitor activity in the heated plasma was measured, and compared with the inhibitor titer of patient's plasma without pre-heating.*

| Test material Patient's citrated plasma | Inhibitor activity Units per ml |
|---|---|
| Without heating | 102 |
| Heating to 65° C for 10 min. | 34 |
| —»— » 70° C for 10 min. | 22 |

The inhibitor had a relatively high heat stability, see Table V. After heating of diluted plasma to 70° C for 10 min., it still contained about 1/5 of its original inhibitor activity.

## DISCUSSION

The risk of developing circulating inhibitor against the plasma deficiency factor in hemophiliacs seems to be relatively low. In Norway such inhibitor has been diagnosed in only two cases, in connection with a material of more than 100 patients with hemophilia A. In both these cases the inhibitor was

detected at a time when the patient had no bleeding at all. Usually the inhibitor in a hemophiliac is discovered because the patient is found to be refractory to transfusion therapy during a bleeding episode. But the inhibitor would probably not increase the bleeding tendency in a patient with a supposed zero AHA level, as it would then have no substrate for its action. This would be in accordance with the two mentioned cases, as the patients have had no aggravation of their bleeding tendency after apperance of the inhibitor.

The published cases of hemophilia with circulating inhibitors seem to have been originally of the severe deficiency type. The simplest explanation would then be that transfusions have initiated the production of antibodies against the lacking protein factor, though it is in many ways difficult to classify the reaction as a pure immunological one.

An assumption that a patient with the severest type of hemophilia A has no AHA at all in plasma, is difficult to confirm from the results of ordinary factor assaying. In 19 cases of grave hemophilia A, the plasma AHA levels were assayed by the author with the described one-stage cephalin system, and in all these cases AHA was found below one per cent of normal average. The assay technique, however, cannot with certainty differentiate between smaller changes in AHA at very low levels. The system shows that severe hemophiliacs of type A have roughly the same degree of clotting defect; it cannot decide definitely whether this defect is total or not. Investigators using an assay system based on the thromboplastin generation test (Pool & Robinson 1959) have indicated that there is appreciable AHA activity in the plasmas o patients with severe classical hemophilia.

In the present case, the cephalin time o the patient's plasma was not found to in crease significantly when the inhibitor deve loped. Addition of patient's inhibitor plasm to his non-inhibitor plasma did not chang its cephalin time. These observations, an the fact that the patient's hemorrhagic dia thesis did not become more severe than be fore his last transfusions, indicate that hi plasma really was totally devoid of AHA beforehand. That his plasma *after* the deve lopment of inhibitor must have been empt of AHA, seems evident, considering th strength of the inhibitor activity. Consisten with the results of mixing of samples of th patient's plasma batches, addition of sma amounts of inhibitor plasma did not chang the cephalin time of the plasmas from ord nary patients with severe hemophilia A. Th seems then to support the general assumptio that such plasmas contain no AHA at al which would be in good accordance with th fact that the patients are specially liable t develop AHA inhibitor after transfusions.

The inhibitor of the described patient d creased the AHA levels in partially AH deficient plasmas from patients with mil hemophilia A, and from patients with vo Willebrand's disease, in the same way as decreased AHA in normal plasma. Th shows that the inhibitor cannot distinguis between the AHA protein in normal plasm and in plasma from the cases with partial d ficiencies. The AHA protein in those grou may of course still be of a somewhat differe type, although the attempt to separate the with specific inhibitor did not succeed.

As to the assaying of the strength of th

AHA inhibitor, this is an important problem, but involves great difficulties. All measuring of intrinsic clotting factors is necessarily complicated, and a real measuring of inhibitor activity implies a comparison of two estimations of AHA, giving the effect of the inhibitor over a certain time period on AHA in an indicator material. The author has felt it to be of importance in a general assay of AHA inhibitor activity, to use a normal human plasma as indicator of the effect. In this way, the results should be directly applicable in the reasoning in connection with ordinary blood and plasma transfusions. Some animal AHA materials may give certain advantages as indicators, being *e. g.* easier to supply in higher concentrations, and giving a convenient standard for comparisons over longer time periods. But such advantages can hardly compensate for the difficulties of having to compare the effect of the inhibitor on AHA from different species. If, however, the results of the assay are going to be used in connection with transfusions with animal AHA concentrates, then of course the inhibitor activity directed just against such factor material is the right effect to measure.

In accordance with the findings of several other investigators, the inhibitor activity in the present patient's plasma was found to decrease when the patient was controlled in the months after the first appearance, during which time he got no new transfusions.

The results of the investigations confirmed the findings of other workers that the inhibitor is found in high titers also in serum and in plasma adsorbed with aluminum hydroxide or barium sulphate, and that the inhibitor is not dialyzable and relatively resistent to heating. The inhibitor seems also to be relatively stable during storage of plasma at —20° C.

## SUMMARY

A case is presented of acquired circulating inhibitor against factor VIII (AHA) in a 29-year old man with hemophilia A. The inhibitor was discovered in a plasma sample prepared 82 days after transfusions with blood and plasma. Direct comparison could be done between this inhibitor plasma and plasma without inhibitor, prepared in the same way before the transfusions. The patient's bleeding tendency was not aggravated after the transfusions.

The cephalin time of the inhibitor plasma was not significantly prolonged compared with patient's non-inhibitor plasma. Addition of small amounts of inhibitor plasma to patient's non-inhibitor plasma did not significantly change its cephalin time, nor did such addition change the cephalin time of plasmas from other patients with severe hemophilia A.

A method of assaying the strength of AHA inhibitor activity is presented, as a modification of the assay of Biggs & Bidwell (1959).

The inhibitor activity was found to grow gradually weaker in plasma samples at later controls of the patient.

The inhibitor could be shown to work also against AHA in plasma from ordinary hemophiliacs with partial deficiency of AHA, and from patients with von Willebrand's disease.

The inhibitor was not dialyzable, and could be demonstrated both in serum and in adsorbed plasma. The inhibitor was relatively

heat resistent and stable in plasma stored at —20° C.

The question is descussed whether patients with severe hemophilia A ordinarily are completely devoid of AHA protein in their plasma. The results of the presented investigations seem to support such an assumption.

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# THE EFFECT OF THYROID FUNCTION ON BONE MARROW DEHYDROGENASE ACTIVITY

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In recent years colorimetric determination f dehydrogenase activity by means of tetra-olium salts has been used to elucidate the unctional response of tissues to injury and o the influence of hormones in animals Hershey, Cruickshank & Mullins, 1958, versen, 1959 and Myren, 1960).

In these animal studies, thin slices of skin, ver and spleen, and small bits of bone arrow were incubated with an isotonic olution of 1 per cent, 2, 3, 5-triphenyltetra-olium chloride (TTC) in phosphate buffer or one hour at 37° C. The colorless TTC as reduced by the liberated hydrogen ions to red-colored formazan, the deposition of hich in the cell cytoplasm was proportional o the dehydrogenase activity. The formazan as then extracted with acetone for 24 hours, nd the color intesity determined per mg dry eight of tissue. The error of method was und to be 7 to 10 per cent.

The experimental results in the animal ries have shown that the dehydrogenase tivity of liver and hematopoetic tissue may influenced in similar ways by different ses of carbon tetrachloride and by adreno-cortical function. An elevation of bone marrow dehydrogenase activity was caused by the administration of ACTH.

The method has also been invoked to study tissue reactions to the injurious effect of diseases in man (Mitsuhashi, Saito, Yamagushi, Salvai, Yoshimura & Tizuka, 1958, and Myren, 1960). Thus, in a preliminary report the colorimetric determination of dehydrogenase activity was carried out in bone marrow specimens from 58 patients (Myren, 1960). Only one of these had thyreotoxicosis. A marked elevation of dehydrogenase activity was found in the bone marrow, suggesting that the thyroid may influence the hematopoetic tissue through dehydrogenases.

This question is closely connected with the problem of the hormonal regulation of enzymatic activities in man, and especially with the role of the thyroid for the function of hematopoetic tissue. Therefore the present report is concerned with the relation between the bone marrow dehydrogenase activity and the thyroid function as tested by the metabolic rate (BMR) and the radioactive iodine uptake ($I^{131}$).

Table I. *Number of patients in relation to age and BMR.*

| BMR | Age in years 20—40 | 41—50 | 51—60 | 61—70 | 71— | Number of patients |
|---|---|---|---|---|---|---|
| 130 per cent and higher, | | | | | | |
| Females..... | 4 | 8 | 5 | 3 | 1 | 21 |
| Males ..... | | | 1 | 2 | | 3 |
| below 130 per cent, | | | | | | |
| Females..... | 3 | 5 | 6 | 5 | 3 | 22 |
| Males ..... | 1 | | 2 | 1 | 1 | 5 |
| Total number of patients | 8 | 13 | 14 | 11 | 5 | 51 |

## MATERIAL AND METHODS

The material consisted of 51 patients, the age ranging from 20 to 78 years (Table I). Of these cases 43 were females and 8 males.

BMR was determined by a Krogh apparatus in the morning before breakfast. In each patient the examination was repeated twice, and the mean values were recorded as BMR.

In 24 patients the BMR was 130 per cent or higher (high values). In 23 of them a clinical diagnosis of thyreotoxicosis was made. In addition one woman had glycosuria and one had hypertonia. One woman had paroxysmal atrial fibrillation and one a slight sideropenic anemia.

In 27 patients the BMR was below 130 per cent. The distribution of these cases in age groups was as in the groups of patients with high values of BMR (Table II). Of these 27 patients, 14 were treated with neomarcazol for thyreotoxicosis, one had thyroiditis and one an operated pituitary adenoma. The other cases (11 patients) were observed for hypo- or hyperthyreosis. In three of them hypothyrcosis was found. In the other 8 cases an abnormal thyroid function was clinically not stated. One of them had hypertonia and one diabetes mellitus.

Thus, the two groups of patients were comparable but for the BMR. Also the average values of hemoglobin concentration and leukocyte count peripheral blood were not significantly different.

$I^{131}$-uptake of the thyroid gland was determin 24 hours after peroral administration of 12 to micro C of radioactive iodine. In 15 patients t uptake of $I^{131}$ was higher than 65 per cent, indi ting hyperthyreosis, and in 9 cases the uptake w lower.

The correlation of the BMR to the values $I^{131}$-uptake (Fig. 3) showed that the patients w a BMR of 130 per cent or higher had an uptake $I^{131}$ higher than 65 per cent. One case only lower values. In the patients with BMR below per cent the values of $I^{131}$-uptake were lower th 65 per cent.

A good correlation was thus found between BMR and $I^{131}$-uptake of thyroid gland. In present series of patients a BMR of 130 per c or higher was found in nearly all cases with thyr toxicosis.

Bone marrow specimens were obtained by usual sternal puncture in local anesthesia. The b marrow particles only were used for examinati For morphological study smears were stained the method of Giemsa, and the preparations w examined by routine microscopy. For determinat of dehydrogenase activity the method repor previously (Myren, 1960), was used. Two-third the incubation medium consisted of 1 per cent TTC, and one-third of 0.1 M sodium succinate phosphate buffer (isotonic solution, pH 7.4). A incubation of the bone marrow specimens for hour at 37° C, the red-colored formazan was

Table II. *Number of patients in age groups.* $I^{131}$*-uptake.*

| $I^{131}$-uptake | Age in years 20—40 | 41—50 | 51—60 | 61—70 | 71— | Num of patie |
|---|---|---|---|---|---|---|
| Females..... | 5 | 9 | 6 | 3 | | 23 |
| Males ..... | | | 1 | | | 1 |
| Number of patients .... | 5 | 9 | 7 | 3 | | 24 |

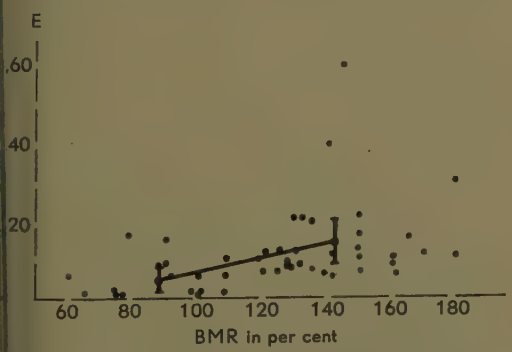
racted with 5 ml of acetone for 24 hours. Also the
at content of the bone marrow specimens was re-
noved by this procedure. The color intensity was
etermined in a Beckman colorimeter, and the de-
ydrogenase activity estimated per mg dry weight
n Beckman units. The weight of the dry marrow
pecimens was between 0.3 and 8 mg, usually be-
ween 1 and 3 mg.

## RESULTS

The morphological picture of erythro-
oetic and leukopoetic cells was not signi-
icantly different in the two groups of pa-
ients examined. On the other hand, the
legree of dehydrogenase activity in bone
narrow specimens from patients with a high
3MR and $I^{131}$-uptake was significantly di-
ergent from that found in patients with
ower BMR and radioactive iodine uptake.

Thus, the *correlation between the de-
ydrogenase activity* and BMR (Fig. 1)
howed the following:

The activity of bone marrow specimens
rom patients with a high BMR (130 per
ent and higher) varied between 0.04 and
.58 Beckman units. The average value of
ctivity was 0.15 and the standard deviation
= 0.026. In these patients the BMR varied




g. 1. The correlation between dehydrogenase
tivity (Beckman units) and BMR in 51 patients.

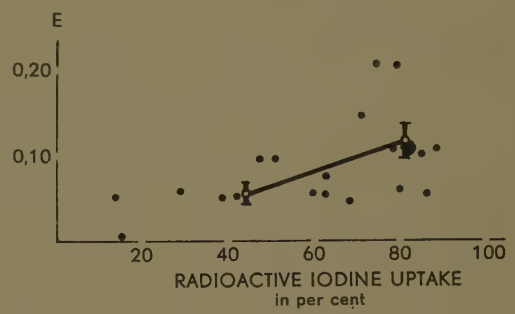



Fig. 2. The correlation between dehydrogenase activity and the uptake of $I^{131}$ in 24 patients.

from 130 to 180 per cent, the average value being 144.

The activity of bone marrow specimens from the group of patients with BMR lower than 130 per cent varied from 0.002 to 0.168 Beckman units. The average value of activity was 0.05 and the standard deviation $\pm$ 0.023. In these cases the BMR varied from 60 to 128 per cent, the mean value being 88 per cent.

The difference in bone marrow dehydrogenase activity between the two groups of patients examined, was found to be statistically significant (p-values below 0.5 per cent also when the two highest values of activity were excluded).

The *correlation of dehydrogenase activity to the uptake of $I^{131}$* is shown in Fig. 2.

In the patients with high $I^{131}$-uptake (above 65 per cent) the average dehydrogenase activity was 0.12 Beckman units as compared to 0.15 in the BMR-group (130 per cent and higher). The standard deviation was 0.017 and the average $I^{131}$-uptake was 81 per cent.

In the patients with $I^{131}$-uptake lower than 65 per cent, the average dehydrogenase activity was 0.055 as compared to 0.05 in

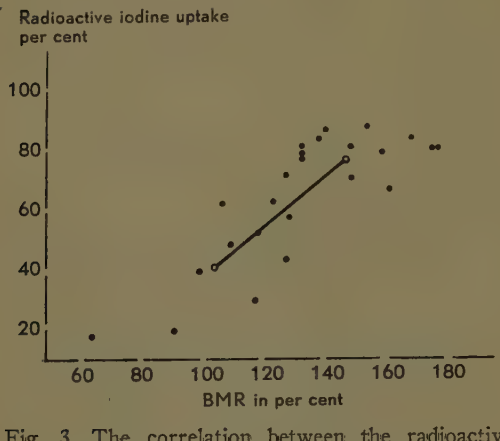



Fig. 3. The correlation between the radioactive iodine uptake and BMR in 24 patients.

the BMR-group (below 130 per cent). The standard deviation was 0.011 and the average value of $I^{131}$-uptake was 44 per cent.

The difference found between the dehydrogenase activity of bone marrow in these two groups of patients was statistically significant (p-value below 0.5 per cent).

## COMMENTS AND CONCLUSIONS

The spreading of individual values of bone marrow dehydrogenase activity was considerable. A significantly higher degree of activity was, however, found in patients with an elevated BMR and $I^{131}$-uptake than in patients with a normal and decreased BMR and radioactive iodine uptake. The finding may indicate that the function of bone marrow may be influenced by the thyroid through dehydrogenase activity, which partakes in the citric acid cycle. Thus, in thyreotoxicosis, the bone marrow function may be high as is the total consumption of oxygen determined by BMR. On the other hand, in myxedema patients, the function may be low, the result of which may anemia.

The finding may also perhaps indic that the determination of bone marrow hydrogenase activity may supply informati on thyroid function.

## SUMMARY

The relation between bone marrow hydrogenase activity and thyroid function measured by the BMR and the $I^{131}$-upta was examined in 51 patients. The anzyma activity was determined by means of 2, 3, triphenyltetrazolium chloride (TTC) [usi a colorimetric method reported previousl

The average dehydrogenase activity v significantly higher in patients with an av age BMR of 144 per cent and an $I^{131}$-upta of 81 per cent than in the patients with average BMR of 88 per cent and an I uptake of 44 per cent. [The finding may dicate that thyroid function is of importa for the degree of bone marrow dehydrog ase activity. The thyroid may thus influe the hematopoetic tissue partly *via* the hydrogenases, which partake in ener yielding processes. On the other hand, amination of bone marrow dehydrogen activity may perhaps provide information thyroid function].

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# BINDING OF HEPARIN TO FIBRIN(OGEN)

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In a previous work, the existence of poorly soluble heparin-fibrinogen complexes was demonstrated (Godal 1960 a). It was found that such complexes formed more easily with denatured fibrinogen. This finding suggests that some heparin may be bound to fibrin during coagulation of fibrinogen with thrombin in the presence of heparin.

As demonstrated below, this hypothesis was confirmed. Further, heparin and fibrin seem to interact in stoichiometric proportions. Finally, it was found that other plasma constituents may interfere with the binding of heparin to fibrin.

## MATERIALS AND METHODS

*Albumin.* A commercial preparation, Albumin "Kabi", Stockholm, Sweden, containing 20 per cent protein was used.

The other materials and methods used in this work have been described previously (Godal 1960 b & c).

## RESULTS

1) *Binding of heparin to purified fibrin(ogen). Stoichiometric relationship.*

A 0.5 per cent solution of purified fibrinogen was diluted serially with buffer-saline. To each dilution, one-ninth volume of heparin (10 units per ml) was added, and the mixtures were then clotted with one-ten volume of thrombin (15 NIH units per m One hour later, the fibrin clots were moved by winding them up on a wood stick, and the heparin content of the supernatant was determined by titration with antiheparin agent, polybrene. As a contr an identical series was run, but one-ten volume of heparin was added to the supernatant *after* removal of the clot. The res is given in Fig. 1. As is evident, nearly the heparin was removed if the fibrinog

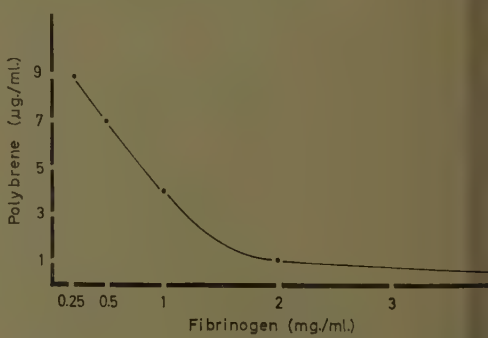


Fig. 1. *The heparin-binding capacity of fibri* The heparin content of the supernatant was det mined by titration in the following clotting syste 0.2 ml citrated, platelet poor plasma, 0.1 ml sup natant, 0.1 ml polybrene solution of various c centrations, 0.2 ml of thrombin. Each point on curve represents a titration curve. For details, text.

Table I. *Heparin-binding capacity of fibrinogen.*

| Precipitation procedure | Polybrene conc. (μg/ml) necessary for neutralization of heparin in supernatant |
|---|---|
| oagulation by heat .... | 1 |
| reeipitation by ethanol. | 0 |
| oagulation by thrombin | 1 |
| ontrol (heparin added to supernatant) ..... | 9 |

'he following mixtures were tested:
.6 ml fibrinogen solution (0.4 per cent),
.2 ml heparin solution (10 units per ml),
.2 ml thrombin solution or physiological saline.

ifter precipitation of plasma fraction I, the supernatant was deprived of its ethanol content by lyophilization, and then reconstituted with distilled vater.

oncentration was 0.2 per cent or higher. 'urther, a stoichiometric relationship between fibrin and heparin is suggested.

If, instead of cloting, heparinized fibrinogen was precipitated by heating to 56° C or 15 minutes, or precipitated by ethanol in ne cold (Cohn, Strong, Hughes, Hulford, Ashworth, Melin & Taylor, 1946), heparin vas bound to the precipitated fibrinogen to ne same extent as to fibrin (Table I).

2) *Effect of albumin on the heparin-binding capacity of fibrinogen.*

A weak, inhibitory effect of albumin (final oncentration: 2 per cent) on the heparin-brinogen interaction was observed (Tab- II).

3) *Heparin-fibrinogen interaction in whoplasma.*

Due to the antithrombin effect of heparin plasma, the binding of heparin was determined by heating heparinized plasma to 56° C for 15 minutes. With this procedure, it could be shown that much less heparin was removed from plasma than from a solution of purified fibrinogen (Table II).

## DISCUSSION

The data presented above suggest that heparin and fibrin(ogen) interact in stoichiometric proportions during coagulation or precipitation of fibrinogen. Based on the assumption that the molecular weight of heparin is 16,500 (Jensen, Snellman & Sylvén 1948), and that of fibrinogen about 350,000 (Katz, Gutfreund, Shulman & Ferry 1952), at least ten fibrin(ogen) molecules are necessary to bind one heparin molecule. This ratio is very similar to that found for precipitation of fibrinogen by heparin in the cold (Godal 1960 a), and probably suggests that an aggregation of fibrin(ogen) molecules

Table II. *The effect of plasma proteins on the heparin-binding capacity of fibrogen.*

| Material tested | Polybrene conc. (μg/ml) necessary for neutralization of heparin in supernatant |
|---|---|
| Fibrinogen..... | 1 |
| Fibrinogen + Albumin.. | 2 |
| Plasma ..... | 6 |
| Control (heparin added to supernatant (plasma)) | 9 |

The following mixtures were tested:
1.6 ml fibrinogen solution (0.4 per cent), or 1.6 ml citrated, platelet poor plasma,
0.2 ml heparin solution (10 units per ml),
0.2 ml albumin solution (20 per cent protein), or 0.2 ml phvsiological saline,

After heating to 56° C for 15 minutes, the supernatants were tested for their heparin content by titration with polybrene.

takes place prior to the binding of heparin. It is interesting to note that heparin precipitates together with fibrin(ogen), irrespective of the means by which the precipitation is carried out.

The weak heparin binding effect of fibrinogen in plasma suggests a competitive relationship between fibrin(ogen) and other plasma constituents for heparin. Such a relationship might probably explain why very small amounts of heparin prolong the thrombin time of purified fibrinogen, but are ineffective in whole plasma (Shinowara 1949, Godal 1961).

Finally, the binding of heparin to fibrin(ogen) might also contribute to the understanding of the antithrombin effect of heparin under various experimental conditions. Thus, whenever the tendency to aggregation of fibrin(ogen) is increased, the antithrombin effect of heparin is reduced (Godal 1961). From what is said above, the speed of the aggregation process must influence the heparin amount available for inactivation of thrombin.

## SUMMARY

1. Heparin is bound to fibrin during coagulation of fibrinogen by thrombin, and heparin and fibrin interact in stoichiometric proportions. Two milligrams of fibrin bind about 10 micrograms of heparin.

2. If heparinized fibrinogen is precipitat by heat, or by addition of ethanol in the co heparin is similarly bound.

3. Albumin, and still more other plasm constituents, inhibit the binding of hepar to fibrin(ogen).

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# EFFECTS OF HYPOTHALAMIC STIMULATION ON ERYTHROPOIESIS IN RABBITS

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The results of several earlier experimental studies in animals suggest that a regulatory influence on erythropoiesis may be exerted from the hypothalamus. The subject has been reviewed by Beer (1942), Wespi (1944), Komiya (1956), and others. However, the techniques used in these earlier experiments have been relatively crude, and none of them has been accepted as conclusive. The hypothalamus has been stimulated by injections of silica, silver nitrate, or by "Hirnstich". Wespi (1944) and Linke (1958) have applied electrical stimulation with electrodes in the hypothalamic region, but only in acute experiments. They report some changes in the hemoglobin percentage and the number of erythrocytes (Wespi) or in the number of reticulocytes (Linke). The changes were observed within a few hours and therefore can not be attributed to formation of new red blood cells.

An increase in hemoglobin and erythrocytes has been produced by lesions in the region of the tuber cinereum (Komiya and Hayashida, *cit.* Komiya 1956). Furthermore, several investigators have found that transection of the spinal cord in the cervical or upper two thoracic segments prevents the reticulocytosis following bleeding (Heilmeyer, 1933; Somogyi, 1938; Nasu, 1937; Kimura, 1943), or exposure to low oxygen tension (Hayashida, 1936; Sugiura, 1938). These observations suggest that cerebral structures may influence erythropoiesis by impulses passing down the spinal cord and leaving the latter in the cervical or upper thoracic region.

In none of these earlier studies have measurements been made of the blood volume or the total red cell mass. Such measurements are necessary in order to demonstrate whether a real polyglobulia is present or not.

The aim of the present investigation has been to study the effect of hypothalamic electrical stimulation on erythropoiesis in chronic experiments and by means of more adequate hematological methods, including measurements of the total red cell mass. It is shown that the hypothalamus may influence the rate of red blood cell production, as indicated also by previous clinical findings

Table I. *Results of repeated red cell mass (RCM) determinations in a control series of nine rabbits.*

| Control No. | Initial RCM, ml | Second RCM, ml | Difference ml | Difference per cent |
|---|---|---|---|---|
| 1 | 39.0 | 40.8 | +1.8 | +4.6 |
| 2 | 45.6 | 44.4 | —1.2 | —2.7 |
| 3 | 36.4 | 37.6 | +1.2 | +3.3 |
| 4 | 45.5 | 43.8 | —1.7 | —3.7 |
| 5 | 77.0 | 73.2 | —3.8 | —4.9 |
| 6 | 51.5 | 54.7 | +3.2 | +6.2 |
| 7 | 102.0 | 97.9 | —4.1 | —3.9 |
| 8 | 59.4 | 62.3 | +2.9 | +4.9 |
| 9 | 70.4 | 67.3 | —3.1 | —4.3 |

The variance of the differences in ml is 3.81 with a standard deviation of 1.95. The confidence limits (95 per cent band) for any given estimation (e) of RCM = e $\pm$ 2 $\times$ 1.95.

(Seip, 1953). A preliminary report has appeared (Seip, Halvorsen, Andersen & Kaada, 1960).

## METHODS

The conclusions are based on observations from 28 adult male and female (non-pregnant) rabbits in which complete data were obtained. All these animals had normal blood picture when the experiments were initiated.

Before implantation of the electrodes the following parameters were determined: total red cell mass, reticulocytes, hematocrit, hemoglobin, red blood cells, white blood cells, and differential count. Blood from the ear veins was used for all determinations. Blood for red cell mass and hematocrit determination was taken by venipuncture, the other samples by pin-prick.

Reticulocytes were stained with brilliant cresyl blue according to Seip (1955). In order to reduce the counting error 5,000 red cells were ordinarily counted in each preparation, but only 2,000 when the number of reticulocytes was especially high.

The red cell mass (RCM) was determined with the $Cr^{51}$ method (Gray & Sterling, 1950, Donohue *et al.*, 1955). The method was adapted for use in rabbits. Five to six ml blood was withdrawn from an ear vein into a heparinized syringe, centrifuged at 1,500 r.p.m., the plasma pipetted off and the red cells incubated with 3 to 4 $\mu$C $Cr^{51}$ [1]) at room temperature for 45 minutes. The red cells were washed three times with normal saline and resuspended in the original plasma and additional saline making the volume 6 ml. An aliquot of this was weighed and taken for counting. The remaining was injected into an ear vein, the amount being determined by weighing the syringe before and after the injection. Twenty minutes following the injection of the tagged cells 1.0 to 1.5 ml blood was withdrawn for counting and the hematocrit determined in this sample. Only one sample was used in order to minimize the loss of blood from the rabbits. The error of the hematocrit determination due to trapped plasma was assumed to be constant and was disregarded. The samples were hemolyzed with distilled water making the volume 3 ml and counted in a well type scintillation counter[2] with an efficiency of $1 \times 10^6$ c.p.m. per microcurie of $I^{131}$ with a gamma crystal and a background of less than 12 c.p.m. Calculation:

$$\text{RCM} = \frac{\text{total counts in circulation}}{\text{counts per ml packed red cells.}}$$

The accuracy of the method was tested by second red cell mass determination with $Cr^{51}$ within 24 hours of the first in nine rabbits. Immediately before the second RCM determination blood was withdrawn for counting and the remaining activity calculated per cc packed red cells. The results of the two determinations agreed within 4.1 % (Table I).

The other blood studies were performed with conventional clinical methods. A micro-method was used for hematocrit determination.

Following these measurements, needle electrodes were implanted in the hypothalamic region under urethane-chloralose anesthesia. The stereotaxic coordinates published by Sawyer, Everett & Green

[1]) $Na_2Cr^{51}O_4$ supplied by The Radiochemical Centre, Amersham, England.

[2]) SC-51 Autoscaler, Tracerlab Inc., Boston Mass.

(1954) served as a guide in the placement of the electrodes. Three to seven days later stimulation was started. The non-anesthetized animals were stimulated about four hours daily for fourteen days. The stimulus parameters were: square wave pulses of a frequency of 20/sec., 1 msec.' duration and 0.5—10 volts. The animals were stimulated 10 seconds every 20th second. During stimulation the animals' respiration was observed, and in four of the positive experiments the effect of stimulation on the blood pressure was recorded at the termination of the experiment.

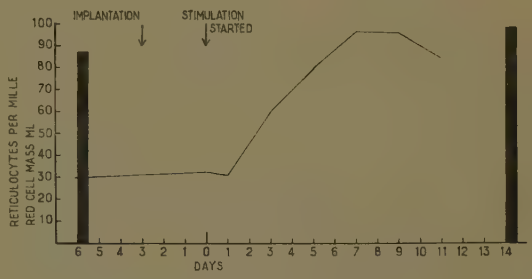
Immediately before and every other day during the stimulation period reticulocyte, white blood cell and differential counts were performed. At the end of the stimulation period hemoglobin, erythrocytes, hematocrit and total red cell mass were again determined. The hemoglobin and erythrocyte determinations were performed in our routine laboratory and showed, as expected with these relatively crude methods, no significant changes. They will not be considered in the following discussion. — The animals were then killed, and the localization of the electrodes was identified histologically. The organs were examined to exclude any gross bleeding. All animals were weighed before, during and after the stimulation periods.

Many of the animals had poor appetite for some days after implantation of the electrodes, and several showed decreased appetite and activity throughout the stimulation periods. A few animals died under or within the next few days following the implantation.

## RESULTS

### *Red blood cells*

*A. Positive experiments.* In eight of the 28 experiments a sustained and sometimes pronounced reticulocytosis and a significant increase in the red cell mass occurred. These experiments were recorded as definitely positive. The most important data for each experiment are listed in Section A of Table II. Fig. 1 illustrates an experiment where a particularly marked increase in erythropoietic activity could be demonstrated.




RETICULOCYTES (——) AND RED CELL MASS (▮) IN ONE OF THE RABBITS SHOWING A POSITIVE RESPONSE

The *reticulocytosis* observed in the eight positive experiments was of varying degree (Fig. 2). From initial values between 2.26 and 3.38 per cent, the reticulocytes increased up to levels varying from 4.78 to 10.05. The highest values were usually found after about one week's stimulation.

The *increase in red cell mass* varied from 4.1 to 14.9 ml, *i. e.* from 7.2 to 27.2 per cent of the initial level. From Table II it is apparent that the increase in red cell mass can not be explained by an increase in body weight. Three of the eight animals lost weight during the experiment, and only one (No. 32) showed a more pronounced gain in weight. However, in this animal the increase in red cell mass was relatively much greater than was the increase in weight.

The *hematocrit* showed a moderate rise in seven of the eight positive animals and remained unchanged in one. The mean value increased from 35.1 to 38.1.

*B. Negative responses.* Ten rabbits showed no erythropoietic response (Table II, B, and Fig. 3). In these cases no or only a minimal reticulocytosis occurred, and a decline was found in the red cell mass. The

Table II. *Hematological data.*

| Rabbit No. | Body weight (g) | | Red cell mass (ml) | | Change in red cell mass | | Reticulocytes (per cent) | | Hematocrit | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Before implantation | After stimulation | Before implantation | After stimulation | ml | per cent | Before implantation | Highest value after stimulation | Before implantation | After stimulation |
| A. Definitely positive response | | | | | | | | | | |
| 13 | 3600 | 3200 | 55.9 | 63.0 | + 7.1 | +12.7 | 2.26 | 4.78 | 35 | 39 |
| 22 | 3050 | 3120 | 57.0 | 61.1 | + 4.1 | + 7.2 | 2.84 | 5.20 | 37 | 40 |
| 23 | 3990 | 3720 | 87.2 | 98.1 | +10.9 | +12.5 | 3.02 | 9.65 | 44 | 45 |
| 24 | 2580 | 2600 | 41.2 | 52.4 | +11.2 | +27.2 | 2.86 | 10.05 | 40 | 40 |
| 32 | 3300 | 3750 | 48.4 | 59.7 | +11.3 | +23.3 | 3.38 | 5.70 | 30 | 32 |
| 35 | 3450 | | 55.1 | 60.9 | + 5.8 | +10.5 | 2.50 | 6.35 | 29 | 36 |
| 44 | 3360 | 3200 | 46.3 | 54.0 | + 7.7 | +16.6 | 2.40 | 5.80 | 32 | 35 |
| 47 | 3580 | 3610 | 62.4 | 77.3 | +14.9 | +23.9 | 2.90 | 5.30 | 34 | 38 |
| B. Negative response | | | | | | | | | | |
| 3 | 3300 | 2800 | 50.4 | 42.8 | — 7.6 | —15.1 | 2.40 | 3.44 | 40 | 37 |
| 4 | 2350 | 2350 | 30.1 | 23.0 | — 7.1 | —23.6 | 1.92 | 2.38 | 36 | 34 |
| 6 | 3000 | 2800 | 46.9 | 41.6 | — 5.3 | —11.3 | 2.04 | 2.74 | 37 | 33 |
| 9 | 2070 | 1870 | 34.2 | 26.3 | — 7.9 | —23.1 | 2.54 | 2.58 | 40 | 38 |
| 12 | 2770 | 2550 | 49.4 | 42.7 | — 6.7 | —13.6 | 2.54 | 3.96 | 41 | 37 |
| 14 | 2860 | 3060 | 46.6 | 43.1 | — 3.5 | — 7.5 | 2.42 | 3.92 | 36 | 34 |
| 16 | 3100 | | 47.9 | 40.8 | — 7.1 | —14.8 | 3.56 | 3.46 | 36 | 35 |
| 21 | 3710 | 2630 | 97.0 | 70.8 | —26.2 | —27.0 | 2.66 | 3.00 | 43 | 42 |
| 46 | 2380 | 2005 | 51.8 | 43.4 | — 8.4 | —16.2 | 3.78 | 2.60 | 37 | 42.5 |
| 48 | 3850 | 3600 | 61.7 | 53.8 | — 7.9 | —12.6 | 3.18 | 3.98 | 35.5 | 34 |
| C. Slightly positive or doubtful response | | | | | | | | | | |
| 8 | 3600 | 3590 | 54.9 | 53.6 | — 1.3 | — 2.4 | 2.02 | 4.62 | 33 | 34 |
| 17 | 2310 | 2170 | 32.5 | 38.3 | + 5.8 | +17.8 | 2.86 | | 38 | 43 |
| 26 | 2040 | 1810 | 28.3 | 26.3 | — 2.0 | — 7.1 | 3.04 | 8.80 | 39 | 39 |
| 28 | 3650 | 3140 | 61.1 | 64.7 | + 3.6 | + 5.9 | 3.08 | 4.90 | 38 | 35 |
| 33 | 3000 | 3270 | 43.6 | 48.0 | + 4.4 | +10.1 | 3.32 | 4.80 | 31 | 30 |
| 34 | 3100 | 3220 | 52.6 | 61.6 | + 9.0 | +17.3 | 3.35 | 3.38 | 37 | 36 |
| 38 | 2300 | 2200 | 38.6 | 38.5 | — 0.1 | — 0.3 | 2.72 | 7.95 | 39 | 34 |
| 39 | 2750 | 2680 | 41.2 | 45.6 | + 4.4 | +10.7 | 3.54 | 4.72 | 32.5 | 35 |
| 42 | 2250 | 2180 | 34.6 | 34.4 | — 0.2 | — 0.6 | 3.86 | 6.20 | 33.5 | 35 |
| 43 | 3800 | 3530 | 55.5 | 53.0 | — 2.5 | — 4.5 | 2.08 | 8.45 | 35.5 | 33 |

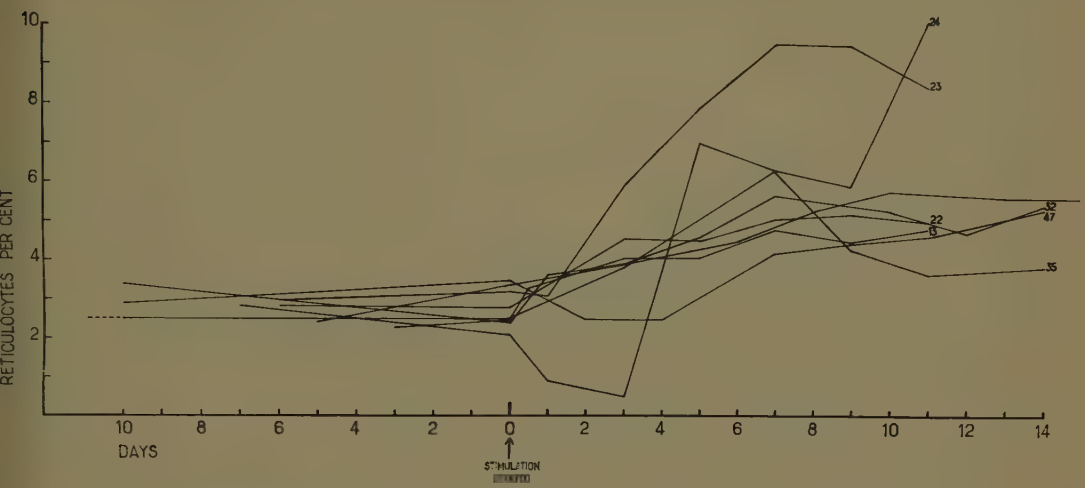



Fig. 2. Reticulocyte curves of the rabbits showing a positive response. Note no effect of implantation of the electrodes three to seven days prior to stimulation.

fall in red cell mass varied from 3.5 to 8.4 ml in nine of these animals, while in one animal with an exceptionally great weight loss there was a decrease in red cell mass amounting to 26.2 ml. The hematocrit decreased in nine of these ten experiments.

*C. Slightly positive or doubtful responses.* Ten animals showed a partial or doubtful response (Table II, C, and Fig. 4). They will be considered in greater detail in the discussion.

*White blood cells*

No characteristic alterations in the total number of white blood cells were recorded. Usually there were only moderate fluctuations from day to day, except in two animals which suffered from wound infection. The differential counts remained relatively unchanged in 20 animals during the entire period of the experiment, while in eight animals a certain neutrophilic granulocytosis with lymphopenia was recorded. Two of these

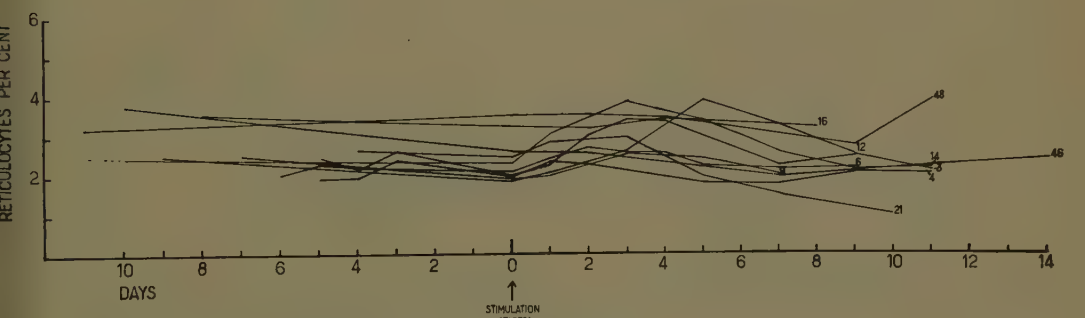



Fig. 3. Reticulocyte curves of the rabbits showing a negative response.

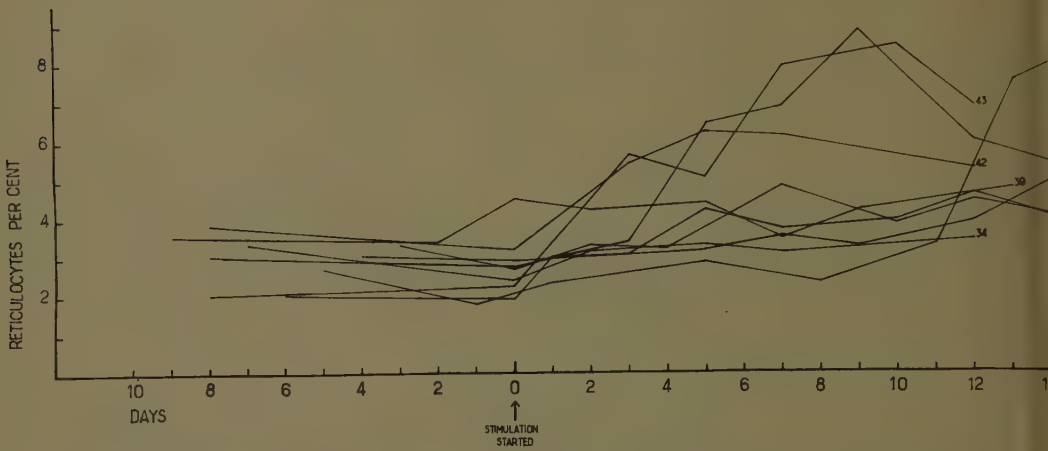



Fig. 4. Reticulocyte curves of the rabbits showing a slightly positive or doubtful response.

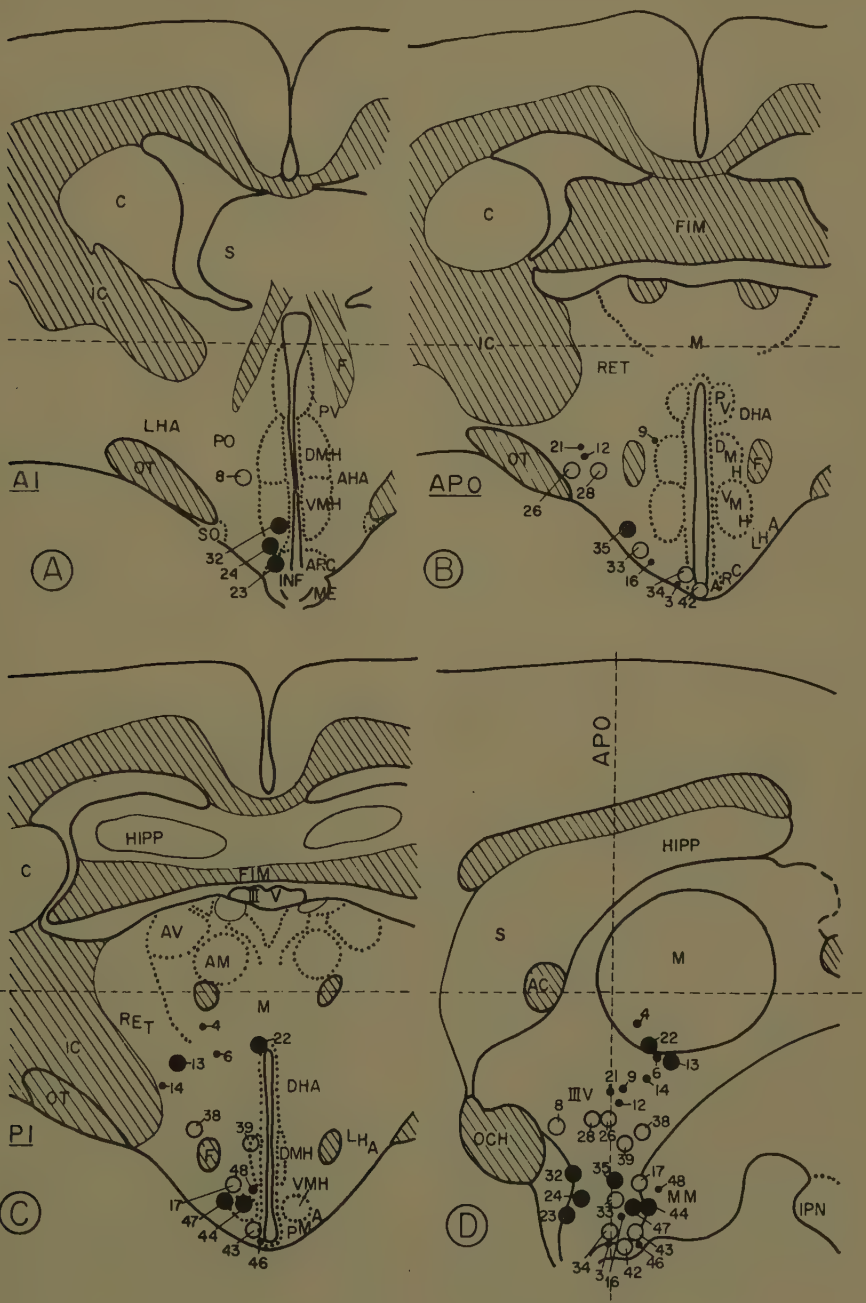
eight animals showed positive, one negative and five slight or doubtful erythropoietic responses.

*Anatomical data*

The localization of the electrodes appears from Fig. 5. The filled circles indicate the electrode sites in the eight animals with marked reticulocytosis as well as a significa increase in red cell mass. The dots indica the negative points, and the open circles t slightly positive or doubtful responses. A positive points are situated in the hypoth lamus, except for two of the weaker positi

Fig. 5. Filled circles, localization of points from which a sustained reticulocytosis as well as a significant increase in red cell mass were obtained by electrical stimulation in the rabbit. Dots, no such effects. Open circles, doubtful responses. *A*—*C*. Transverse sections through the planes A1, APO and P1. In *D* all points are projected to the mid-sagittal plane. Abbreviations mainly according to Sawyer, Everett & Green (1954).

AHA — Anterior hypothalamic area.
AM — N. anteromedialis.
ARC — N. periventricularis arcuatus.
AV — N. anteroventralis.
C — N. caudatus.
DHA — Dorsal hypothalamic area.
DMH — N. dorsomedialis hypothalami.
F — Fornix.
FIM — Fimbria.
HIPP — Hippocampus.
IC — Internal capsule.
INF — Infundibulum.
IPN — N. interpeduncularis.
LHA — Lateral hypothalamic area.
M — Massa intermedia.
ME — Median eminence.
MM — N. mammillaris medialis.
OCH — Optic chiasma.
OT — Optic tract.
PMA — Premammillary area.
PO — Preoptic area.
PV — N. paraventricularis.
RET — N. reticularis.
SO — N. supraopticus.
S — Septum.
VMH — N. ventromedialis hypothalami.
III V — Third ventricle.


A
A1
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S
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AHA
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points which were located in the adjacent ventral part of the thalamus (Nos. 13 and 22). The experiments are too few to allow a more precise localization of the positive area to any specific hypothalamic region.

## DISCUSSION

From the experiments reported it may be concluded that an increased erythropoietic activity with a sustained reticulocytosis and an increase in the total red cell mass can be produced by electrical stimulation of the hypothalamus in rabbits. In the eight definitely positive experiments the reticulocyte curves, red cell mass determinations and hematocrit readings were in good agreement. Of these data the red cell mass and reticulocyte values are the most reliable. The hematocrit readings may be influenced by changes in plasma volume and by variations in the body hematocrit/venous hematocrit ratio. In several animals the increase in red cell mass was considerable, up to 27 per cent of the initial value. This is a high figure in relation to the relatively short stimulation period. These results can not be explained merely by an outflow of reticulocytes already present in the bone marrow. A real increase in erythrocyte formation must have taken place.

The implantation of the electrodes *per se* three to seven days prior to stimulation had no detectable erythropoietic effect. In the positive experiments the reticulocyte level did not rise until stimulation was started. Neither can the rise be accounted for by the blood loss during the implantation and the blood samplings. This loss was kept at a minimum and amounted to only a few milliliters in each animal. As hypoxia is known to st mulate erythropoiesis, the respiratory mov ments were closely watched during the s mulation but no changes were observed. T blood pressure was recorded in four of t positive experiments. No significant alter tion was noted following hypothalam stimulation.

In the ten animals showing a negati response the reticulocyte curves, red cell ma determinations and hematocrit values al agreed well (Table II, B). The decrease red cell mass in these cases can be explain by the blood loss during implantation a blood samplings, not compensated for by i creased erythrocyte production, and in so animals also by reduced erythropoiesis d to impaired appetite and general conditi during the whole or part of the experimen period.

The interpretation of the ten experimen showing a doubtful erythropoietic respon may be debatable (Table II, C). The ele trodes may have been located a little outsi the most effective area, or the effect m have been reduced by local bleeding mechanical destruction by the needle. Fu thermore, the impairment of the appetite a the general condition, which was obvious many of the animals, especially in the fi days after implantation, may have tended reduce their erythropoiesis and their abil to give a good erythropoietic response. Ho ever, the general condition in the group animals with doubtful responses did not see to be different from the other two grou When comparing the values for the total r cell mass before and after stimulation i inevitable, though small blood loss duri the experiment should be remembered.

Rabbit No. 17 is placed in this group only cause the reticulocyte data are lacking. ie red cell mass and hematocrit increased this animal which probably belongs to e positive response group. Rabbit No. 8 owed a mild reticulocytosis, but not so ich as to produce a rise in red cell mass. ie response most likely could be regarded negative. In rabbits Nos. 28, 33 and 39 ticulocytes as well as red cell mass in- eased moderately. They are listed in this oup because the changes were relatively iall. In rabbit No. 38 low reticulocyte unts were found, except on the two last ys of the experiment, and the red cell mass, expected from the reticulocyte curve, did t increase. Possibly this animal would have ven a positive response if the stimulation d been continued over a longer period of ne.

Out of the total series of 28 rabbits there main four, in which there is an apparent screpancy between the reticulocyte curves d the red cell mass determinations. In ree of these (Nos. 26, 42 and 43) a marked ticulocytosis without concomitant increase red cell mass found. Hemolysis or undisc- ered blood loss are the most likely expla- tions of these findings. The red cell survi- l was determined with radiochromium in e of these animals (No. 43), and was found be reduced. The apparent half survival ie was ten days, in normal controls sixteen ys. The increase in red cell mass without iculocytosis in rabbit No. 39 is difficult to plain. A technical error with one of the thods must be suspected.

The *mechanism* by which the hypothala- is may influence erythropoiesis is un- known. However, a few possibilities should be mentioned. Hypoxia is the fundamental erythropoietic stimulus and leads to in- creased formation of humoral erythropoie- sis-stimulating substances, erythropoietins, which in turn exert their effect on the bone marrow. The oxygen supply to the bone mar- row does not seem to be the dicisive factor (Stohlman, Rath & Rose, 1954). The cells of the hypothalamic area in question may possibly be sensitive to small changes in oxy- gen tension and thus regulate the erythro- poietic activity, in the same manner as the cells of the respiratory centers are sensitive to carbon dioxide and anoxia.

A question of great importance is whether the erythropoietic effect of hypothalamic stimulation is mediated through an increased formation of erythropoietins; in other words, whether the erythropoietin production is subject to cerebral regulation. In an attempt to solve this problem, one of us has studied the erythropoietin content in the plasma of some of the rabbits used in the present ex- periments. In some of the animals with a positive response an increased amount of erythropoietic substances could be demon- strated (Halvorsen, 1961).

The possibility that the erythropoietic effect of hypothalamic stimulation might be mediated *via* the pituitary gland by release of ACTH should be mentioned. Some au- thors have ascribed to the ACTH an ery- thropoietic effect (Simpson, Evans & Ro- senberg, 1959). Furthermore, it is known that hypothalamic stimulation may result in a release of ACTH (Mason, 1958; Hume, 1958). This problem will be discussed in a later communication.

## SUMMARY

Some previous animal experiments suggest that the hypothalamus may exert a regulatory influence on erythropoiesis. However, the techniques used in these experiments have been relatively crude, and the results have not been accepted as conclusive. Therefore, the problem was reinvestigated by means of more modern neurophysiological and hematological methods.

Needle electrodes were implanted in the hypothalamic region of 28 adult male and female rabbits. Before the introduction of the electrodes the following hematological studies were performed: total red cell mass determination by means of radiochromium, hematocrit and hemoglobin determination, reticulocyte, erythrocyte, white blood cell and differential counts.

The animals were then stimulated, without anesthesia, for about four hours daily for fourteen days.

Immediately before and every other day during the stimulation period, reticulocyte, white blood cell and differential counts were performed. At the end of the period hematocrit, hemoglobin, red cells and total red cell mass were again determined.

In eight out of the 28 rabbits a marked reticulocytosis and a significant increase in the red cell mass were observed. The electrodes were situated in the hypothalamus or ventral thalamus. In ten rabbits slightly positive or doubtful responses were obtained, and in ten experiments no significant reticulocytosis or increase in the red cell mass were encountered.

These results indicate that a regulatory influence on erythropoiesis may be exerted from the hypothalamic region.



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# PLASMA ERYTHROPOIETIN LEVELS FOLLOWING HYPOTHALAMIC STIMULATION IN THE RABBIT

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In a previous paper Seip, Halvorsen, Andersen & Kaada, 1961 reported the hematological changes in rabbits following electric stimulation of the hypothalamic region. By such stimulation we were able to induce a sustained and sometimes marked reticulocytosis, and in a number of animals also an increase in red cell mass (RCM). In the discussion we indicated several points still in need of elucidation and suggestions for further investigations.

The main question is; how was the observed effect induced? It may have been brought about by direct nervous influence on the bone marrow or by humoral factors. Various investigators have demonstrated the presence of humoral factors stimulating erythropoiesis, erythropoietins, in experimental and pathologic conditions. (Carnot & Deflandre 1906, Hjort 1936, Bonsdorff & Jalavisto 1948, Reissmann 1950 and Erslev 1953). Pituitary hormones have likewise been claimed to induce erythropoiesis in experimental states (White & Dougherty 1945, Contopoulos, Van Dyke, Simpson, Garcia, Huff, Williams & Evans 1953, Crafts & Meineke 1957, Simpson, Evans & Rosenberg 1959, and Osnes 1960), and pituitary stimulation m possibly account for the reticulocytosis f lowing hypothalamic stimulation. Reticu cyte outflow as the cause of the sustain reticulocytosis is, as discussed in the fi paper, less likely on account of the sim taneous increase in RCM. The mechanis which may be responsible for the reticu cytosis following hypothalamic stimulati can consequently be divided into:

1. Direct nervous influence on the bo marrow.
2. Humoral mechanism.
   a) through specific erythropoietins,
   b) through ACTH or related h mones

though it is possible that more than one these mechanisms operate at the same ti

Does plasma from the stimulated rabb contain an increased amount of fact stimulating erythropoiesis? Is the eryth poietin production subjected to cereb regulation? The purpose of the present pa is to report investigations which attempt answer these questions. The possibility pituitary stimulation as the causative mec nism will be dealt with in a later publicati

## MATERIAL

In order to investigate the various possibilities, asmas from 17 of the stimulated rabbits were amined for their content of erythropoietic stim- ating factor(s). The donor rabbits have been vided into the same groups as in the paper by eip *et al.* 1961. Rabbits responding to hypothalamic imulation with reticulocytosis and increase in CM are in the positive response group, and bbits showing neither reticulocytosis nor increase RCM, are in the negative group. The doubtful sponse group consists of rabbits showing slight inconsistent changes in reticulocytes or RCM.

Plasmas from 7 of the 8 rabbits in the positive sponse group (pos.) were tested. From the doubt- l response group (dbf.) 9 of 10 were tested, but e (K17) was excluded making the number from is group 8, and from the negative response group eg.) two plasmas were tested.

The blood was withdrawn from the donor rab- ts preferably while they were still stimulated or ortly after termination of the stimulation. In 3 bbits the blood was withdrawn during light esthesia (K22, K23 and K24), in the other no esthesia was used. The blood samples were taken a heparinized syringe either by cardiac puncture from auricular veins. The blood was centrifuged, e plasma pipetted off and stored at —5° C. imilarly treated plasma from adult, intact rabbits as used as control.

## METHODS

The current methods for investigating erythro- ietic stimulating factors are crude and at best miquantitative. The problems pertaining to these ethods have recently been reviewed by several thors (Gordon 1959, Erslev 1960, Jacobson, urney & Goldwasser 1960, Linman & Bethell 60, and Reeve, Allen & Roberts 1960). Because the capriciousness of the results and the theoret- al objections to most of the methods, I have ecked some of the results with several methods. The following methods were used:

1. $Fe^{59}$ erythrocyte uptake and reticulocyte counts in starved mice.
2. Reticulocyte counts in intact mice (Osnes 1959).
3. Reticulocyte counts in transfusion-induced polycythemic mice (Jacobson, Marks, Gaston & Goldwasser 1959).

*1. Starved mice.*

In the previously reported studies with starved animals rats have been used as recipients (Fried, Plzak, Jacobson & Goldwasser 1957). In the present study mice were used, and it was necessary to reconsider dosage, number of injections, duration of starvation, interval between $Fe^{59}$-injection and blood sampling and the dose of radioactive iron.

The preliminary investigations showed that starvation rapidly reduced erythropoiesis as noted in rats. After 4 days of starvation, the erythropoiesis as determined by erythrocyte iron uptake was reduced to a minimum, but mortality and the number of spontaneous bleedings were high. Starvation between 54 and 64 hours reduced the erythropoiesis significantly and gave low mortality and small risk of hemorrhage.

In the preliminary experiments intervals between the injection of iron and the final blood samplings of 16 and 24 hours were examined, active plasma from an erythroblastotic infant and a normal human adult plasma being used as control. The difference was greatest when a 24 hour uptake was used.

The plasma to be tested was injected intraperitoneally with insertion of the needle in the midline to minimize the risk of bleeding. Two injections with 48 hours' interval were used. A greater number of injections of whole plasma might possibly produce hemolysis, while one single injection might give too weak response. 0.4 ml plasma was given in each dose, *i.e.* a total amount of 40 ml/kg body weight. The amount of Biuret positive material in each plasma was checked.

The radioactive iron was diluted in an iron-free saline solution to an activity of approximately 0.5 microcurie/ml. 0.10 to 0.20 of this solution was injected intravenously. The amount injected was determined by weighing except in the first series where a volumetric determination was made. Radioactive iron was supplied by the Abbott Laboratories, Chicago, Ill., USA, and a well type scintillation counter[1] with an efficiency of $1 \times 10^6$ c.p.m. per

[1] SC-51 Autoscaler, Tracerlab. inc., Boston, Mass., USA.

microcurie of $I^{131}$ with a gamma crystal and a background of less than 120 c.p.m.

At the end of each experiment hematocrit was measured with a conventional microhematocrit method. Mice with hematocrit readings below 45 (mean — 2 sigma in a control series) were excluded from the study, and so were all showing signs of bleeding.

The blood volume was calculated on the basis of 7 per cent of the weight of the mouse at the time of sacrifice. This figure was reached following determintaion of the blood volume in starved mice with $I^{131}$ tagged albumin.

In each experiment a similar number of mice of the same age and sex were injected with plasma from intact rabbits. Controls were run in each experiment in order to exclude errors induced by differences in schedule, age differences or differences between the individual litters.

The details of the method were as follows: 8 to 9 week-old mice of the White Label, Oslo strain that had been raised in the same cage for at least 4 weeks, were divided at random into two groups, 3 to 5 mice in each group. Both groups continued to live in the same cage. On the first day of the study one group received 0.4 ml of the plasma to be tested intraperitonally, and the other group 0.4 ml of normal rabbit plasma. Feeding was as usual on the first and second day. The morning of the third day both groups were transferred to another cage where they only had water. The same afternoon both groups received the second plasma injection. The afternoon of the fourth day both groups were injected with $Fe^{59}$ in a tail vein, and 24 hours later smears for reticulocyte counts were taken. The animals were anesthetized and blood withdrawn by cardiac puncture for counting and hematocrit readings.

*Statistical analysis.* The sample mean and the standard error of the mean was calculated for each series of the tested plasmas. The difference of the means between the stimulated group and the control group was calculated, and the Student t-test was used to test the significance of this difference. When two series were used to test one plasma, a weighted mean of the differences between the stimulated and the control group was calculated. In series with disproportionate sub-class numbers t significance test was performed with a correcti for disproportion (Snedecor 1956).

The sensitivity of the method was tested comparing an expected active plasma[2] with a norm adult plasma. The response to various dilutions active plasma was tested on 4 groups of mic The first group received undiluted active plasm the second ¾ active and ¼ normal plasma, t third group ½ active and ½ normal plasma a the last group ¼ active and ¾ normal plasm Control groups were injected with the same norm plasma. The results of the tests are shown Table I.

*2. Intact mice.*

11 to 13 week-old mice were used as inta recipients as it was found that the spontaneo variations in the reticulocytes were least in th age group. 0.5 ml plasma was given intraperit neally, and the reticulocytes counted before and and 10 days after the injections.

The sensitivity of the method was tested wi plasmas from two infants with erythroblastosi one (Er 1) with Hb = 5.0 gm and reticulocytes 36 per cent and the other (Er 2) with Hb = 10 gm and reticulocytes = 32 per cent. The resul of these tests are shown in Table II and graphical in Fig. 1.

*3. Polycythemic mice.*

8 to 9 week-old mice were made polycythemic described by Jacobson *et al.* 1959, 0.5 ml plasm was injected intraperitoneally and the reticulocyt counted before and 4 and 10 days after the i jection.

The statistical analysis of the results of re culocyte counts in intact and polycythemic mi was performed using the Student t-test to evalua the difference between the fourth day post-injecti and the pre-injection value.

With all three methods the reticulocyte coun were made on thin smears stained with Nile blu

[1] Plasma from an erythroblastotic infant: H = 5.1 gm. Retic. = 41 per cent.

Table I. *Response to various dilutions of active plasma.*

| Method | Dose | No. of mice | Mean ± s.e. | Diff. of the means | Ratio Er/C |
|---|---|---|---|---|---|
| 59 % uptake | 1/4 Er + 3/4 C | 3 | 17.5 ± 1.3 | | |
| | 1/1 C | 2 | 16.7 ± 1.8 | | |
| | 1/4 Er + 3/4 C | 4 | 33.5 ± 4.1 | 5.6 | 1.2 |
| | 1/1 C | 3 | 24.5 ± 8.7 | | |
| | 1/2 Er + 1/2 C | 5 | 27.7 ± 3.3 | 14.7** | 2.1 |
| | 1/1 C | 3 | 13.0 ± 2.6 | | |
| | 3/4 Er + 1/4 C | 4 | 34.8 ± 2.4 | 18.9** | 2.2 |
| | 1/1 C | 3 | 15.9 ± 3.7 | | |
| | 1/1 Er | 4 | 18.3 ± 6.5 | | |
| | 1/1 C | 4 | 4.9 ± 0.4 | 14.3** | 3.3 |
| | 1/1 Er | 2 | 26.8 ± 7.7 | | |
| | 1/1 C | 2 | 10.8 ± 6.2 | | |
| ticulocytes r mille | 1/4 Er + 3/4 C | 3 | 41.3 ± 15.7 | | |
| | 1/1 C | 3 | 18.5 ± 1.2 | 12.9 | 1.7 |
| | 1/4 Er + 3/4 C | 2 | 33.7 ± 7.5 | | |
| | 1/1 C | 3 | 32.2 ± 20.9 | | |
| | 1/2 Er + 1/2 C | 5 | 32.6 ± 5.5 | 22.3** | 3.2 |
| | 1/1 C | 3 | 10.3 ± 1.0 | | |
| | 3/4 Er + 1/4 C | 3 | 58.2 ± 14.2 | 41.4** | 3.5 |
| | 1/1 C | 3 | 16.8 ± 5.5 | | |
| | 1/1 Er | 4 | 22.9 ± 4.7 | | |
| | 1/1 C | 3 | 6.1 ± 0.9 | 24.4*** | 3.8 |
| | 1/1 Er | 2 | 58.5 ± 4.5 | | |
| | 1/1 C | 1 | 14.5 | | |

= plasma from an erythroblastotic infant with Hb of 5.1 gm and reticulocytes of 410 per mille. C = normal adult plasma. In this and the following tables the asterisks attached to the differences of the means denote e sigificance. Three asterisks indiaate probabilities equal to or less than 0.01, two indicate probalities bee 0.05 and 0.01 and a single one indicates p values between 0.10 and 0.05.

00 erythrocytes were ordinarily counted. When ycythemic mice were used 10,000 erythrocytes re counted. The reticulocytes counts were made a technician who did not know which plasma s injected in the actual series.

## RESULTS

Table III shows the $Fe^{59}$ erythrocyte per nt uptake in starved mice and Table IV e reticulocyte counts in the same recipients. ven plasmas from the positive response group have been tested. Five of these gave a $Fe^{59}$ per cent uptake 10.8 to 18.7 higher than the simultaneously run controls. This difference is in the same range as between the active plasma and control plasma used in the dose response study. Another showed a 6.8 rise while the seventh showed no difference. Three of these differences are significant at a 5 per cent level (K22, K44, K47), and three have p values between 0.05 and 0.10.

Table II.

| Donor | No. of mice | Reticulocytes per mille. | | | Mean diff. ± s.e. (4th day - 0 day) |
|---|---|---|---|---|---|
| | | 0 | 4 | 10 days | |
| Er 1 | 7 | 20.5 | 65.5 | 28.0 | 43.6 ± 6.9*** |
| Er 2 | 5 | 23.4 | 35.2 | 29.8 | 13.8 ± 5.5* |

Reticulocyte response in intact mice after one injection of plasma from erythroblastotic infants on day 0.

From Table IV it is seen that plasmas that gave increased $Fe^{59}$ per cent uptake also produced higher reticulocyte values than the controls; the differences ranging between 8.1 and 26.2 per mille. Four of these differences are significant at a 5 per cent level (K22, K23, K24, K44), in two cases the differences are insignificant, and in one (K32) the statistical analysis showed a significant interaction between the results of the two series and is therefore excluded. When the results with both methods are compared, it is seen that 5 plasmas from the positive response group (K22, K23, K24, K44, K47) showed significant increase with one or both methods.

From the doubtful response group one plasma (K28) showed significant increases in $Fe^{59}$ per cent uptake and reticulocytes when tested in starved mice. The other 7 tested plasmas in this group did not give significant changes. The only plasma from the negative group tested in starved recipients did not increase $Fe^{59}$ per cent uptake or reticulocytes significantly.

Table V shows the results of reticulocyte counts in intact mice. Four plasmas have been tested, one (K44) showing significant and two (K26, K38) slight increases wh the last (K48) showed a decrease in reticu cytes. This effect is illustrated graphically Fig. 2.

Table VI shows the results with po cythemic mice as recipients. Plasma fr K44 and a normal rabbit plasma have b studied.

## COMMENTS

It has previously been pointed out that current methods for determination of e thropoietic stimulating factor(s) are cru and large amounts of the factor(s) m

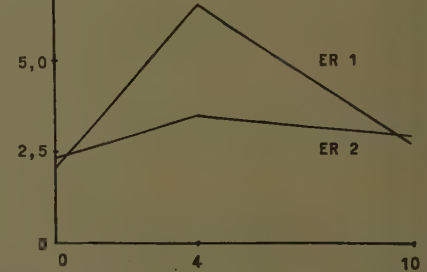


Fig. 1. Reticulocyte response in intact mice a one injection of plasma from two erythroblas infants. See Table I.

Table III. *Per cent uptake of $Fe^{59}$ in the erythrocytes of starved mice.*

| Donor rabbits | | | | | Recipient mice | | | |
|---|---|---|---|---|---|---|---|---|
| | | Reticulocytes per mille. | | | | | | |
| Response group | Rabbit no. | Highest value | Value at the end of stimulation | Biuret gm/100 ml | Series | No. of mice | $Fe^{59}$ % uptake Mean ± s.e. | Diff. of the means |
| Positive (pos.) | K22 | 52.0 | 50.0 | 6.5 | K22 | 4 | 27.0 ± 2.8 | 15.7*** |
| | Control | | | 7.2 | Control | 4 | 11.3 ± 2.2 | |
| | K2 3 | 96.5 | 84.5 | ? | K23 | 4 | 24.7 ± 3.7 | 6.8* |
| | | | | | Control | 5 | 20.3 ± 2.3 | |
| | | | | | K23 | 3 | 21.7 ± 4.8 | |
| | | | | | Control | 3 | 11.3 ± 2.7 | |
| | K24 | 100.5 | 100.5 | 3.5 | K24 | 5 | 33.9 ± 6.1 | 12.3* |
| | | | | | Control | 6 | 21.6 ± 3.8 | |
| | K32 | 57.0 | 54.2 | 3.0 | K32 | 3 | 10.5 ± 1.6 | 10.8* |
| | | | | | Control | 4 | 7.5 ± 1.7 | |
| | | | | | K32 | 2 | 48.0 ± 10.0 | |
| | | | | | Control | 3 | 26.1 ± 8.7 | |
| | K35 | 63.5 | 38.2 | 6.8 | K35 | 3 | 12.4 ± 2.2 | 0.2 |
| | | | | | Control | 3 | 12.7 ± 1.0 | |
| | | | | | K35 | 4 | 5.6 ± 1.5 | |
| | | | | | Control | 3 | 5.0 ± 0.8 | |
| | K44 | 58.0 | 55.5 | 4.9 | K44 | 5 | 42.7 ± 4.3 | 18.7*** |
| | | | | | Control | 5 | 24.0 ± 1.2 | |
| | K47 | 53.0 | 53.0 | 6.5 | K47 | 5 | 32.2 ± 3.8 | 11.2** |
| | | | | | Control | 4 | 25.0 ± 5.3 | |
| | | | | | K47 | 3 | 33.1 ± 6.5 | |
| | | | | | Control | 4 | 16.6 ± 3.0 | |

probably be present in order to give significant responses. With available methods negative responses do not exclude the presence of increased amounts of erythropoietic factors, but may be due to amounts too small to cause significant changes in the recipient animals. This must be taken into consideration in evaluating any study on erythropoietic stimulating factors, and this point may explain many of the controversies in the field.

In the present study both $Fe^{59}$ erythrocyte uptake and reticulocytes have been determined in the starved animals. There is good correlation between these values, but the reticulocytes show greater variation. Controls have been run in each series because small deviations from the schedule influenced the results.

The main objection to the use of starved animals as recipients is that differences in the nutritional content of the test plasmas may give non-specific reactions. For this reason the amount of Biuret positive material was determined. The control plasma showed slightly higher values than the plasmas from the stimulated rabbits. Only one of these

Table III. *(cont.) Per cent uptake of $Fe^{59}$ in the erythrocytes of starved mice.*

| Donor rabbits | | | | | Recipient mice | | | |
|---|---|---|---|---|---|---|---|---|
| Response group | Rabbit no. | Reticulocytes per mille. Highest value | Reticulocytes per mille. Value at the end of stimulation | Biuret gm/100 ml | Series | No. of mice | $Fe^{59}$ % uptake Mean ± s.e. | Diff. of the mean |
| Doubtful. (dbf.) | K26 | 88.0 | 54.0 | 3.5 | K26 | 4 | 14.6 ± 2.0 | |
| | | | | | Control | 3 | 13.7 ± 0.8 | −0.9 |
| | | | | | K26 | 2 | 20.1 ± 3.0 | |
| | | | | | Control | 4 | 23.3 ± 3.2 | |
| | K28 | 49.0 | 49.0 | 6.0 | K28 | 3 | 28.6 ± 7.1 | |
| | | | | | Control | 3 | 10.3 ± 2.5 | 20.2** |
| | | | | | K28 | 4 | 29.5 ± 4.5 | |
| | | | | | Control | 2 | 7.1 ± 1.6 | |
| | K33 | 48.0 | 40.5 | 3.5 | K33 | 4 | 21.2 ± 7.1 | |
| | | | | | Control | 3 | 14.4 ± 0.9 | 8.2 |
| | | | | | K33 | 4 | 17.5 ± 5.5 | |
| | | | | | Control | 2 | 7.5 ± 2.0 | |
| | K34 | 33.8 | 33.8 | ? | K34 | 3 | 9.1 ± 1.6 | |
| | | | | | Control | 2 | 14.4 ± 0.9 | −4.8 |
| | | | | | K34 | 4 | 9.4 ± 3.8 | |
| | | | | | Control | 3 | 13.8 ± 4.9 | |
| | K38 | 79.5 | 79.5 | 6.0 | K38 | 3 | 18.3 ± 5.7 | |
| | | | | | Control | 3 | 15.7 ± 3.5 | 1.7 |
| | | | | | K34 | 2 | 20.1 ± 8.4 | |
| | | | | | Control | 2 | 20.0 ± 7.8 | |
| | K39 | 47.2 | 47.2 | 6.3 | K39 | 5 | 18.7 ± 3.6 | 2.6 |
| | | | | | Control | 4 | 16.1 ± 2.7 | |
| | K42 | 62.0 | 47.5 | 5.0 | K42 | 3 | 30.3 ± 4.6 | |
| | | | | | Control | 3 | 28.9 ± 1.8 | 6.4 |
| | | | | | K42 | 2 | 35.1 ± 5.6 | |
| | | | | | Control | 4 | 23.1 ± 2.2 | |
| | K43 | 84.5 | 68.5 | 4.5 | K43 | 3 | 29.9 ± 7.6 | −0.5 |
| | | | | | Control | 4 | 30.4 ± 3.3 | |
| Negative (neg.) | K46 | 26.2 | 25.5 | 6.0 | K46 | 3 | 37.7 ± 7.4 | 6.0 |
| | | | | | Control | 4 | 31.7 ± 2.1 | |

plasma, K17, had a higher content of Biuret positive material than the control and was for this reason excluded from the study.

The variations of the results with the present method are, as might be expected using a smaller laboratory animal as recipient, somewhat greater than the variations in pre vious studies where rats were used as rec pients (Hodgson, Perreta, Yudilevich Eskuche 1958, Gallagher, McCarthy, Hart Lange 1959). Significance tests on all da are included in this paper. Single great d

Table IV. *Reticulocytes per mille in starved mice.*

| Response group | Series | No. of mice | Mean ± s.e. | Diff. of the means |
|---|---|---|---|---|
| ositive | K22 | 3 | 14.0 ± 1.8 | 8.3*** |
| | Control | 4 | 5.7 ± 0.6 | |
| | K23 | 4 | 28.8 ± 2.6 | 11.7*** |
| | Control | 5 | 17.1 ± 1.0 | |
| | K24 | 5 | 43.0 ± 9.6 | 26.2** |
| | Control | 6 | 16.8 ± 1.5 | |
| | K35 | 4 | 21.3 ± 5.7 | |
| | Control | 3 | 23.2 ± 2.0 | −4.3 |
| | K35 | 4 | 24.5 ± 3.3 | |
| | Control | 3 | 31.2 ± 14.4 | |
| | K44 | 5 | 42.2 ± 10.1 | 25.2** |
| | Control | 5 | 17.0 ± 3.2 | |
| | K47 | 5 | 26.5 ± 5.3 | |
| | Control | 4 | 22.1 ± 5.4 | 8.1 |
| | K47 | 4 | 25.6 ± 5.2 | |
| | Control | 4 | 13.5 ± 2.6 | |
| oubtful | K26 | 4 | 28.5 ± 6.2 | |
| | Control | 1 | 22.0 | 4.5 |
| | K26 | 2 | 29.5 ± 4.5 | |
| | Control | 4 | 26.3 ± 4.8 | |
| | K28 | 3 | 42.0 ± 19.4 | |
| | Control | 3 | 9.3 ± 0.9 | 37.5*** |
| | K28 | 3 | 52·7 ± 14.3 | |
| | Control | 2 | 9.0 ± 1.0 | |
| | K34 | 3 | 7.0 ± 1.8 | |
| | Control | 2 | 13.6 ± 2.4 | 3.9 |
| | K34 | 4 | 30.4 ± 20.6 | |
| | Control | 3 | 19.2 ± 8.0 | |
| | K38 | 3 | 17.2 ± 1.9 | |
| | Control | 3 | 12.0 ± 2.3 | 0.6 |
| | K38 | 2 | 12.3 ± 6.8 | |
| | Control | 2 | 18.8 ± 1.8 | |
| | K39 | 5 | 11.6 ± 2.0 | 2.8 |
| | Control | 4 | 8.8 ± 1.8 | |
| | K42 | 3 | 17.0 ± 3.8 | |
| | Control | 3 | 17.8 ± 3.6 | 6.7 |
| | K42 | 2 | 26.8 ± 0.1 | |
| | Control | 3 | 10.7 ± 5.3 | |
| | K43 | 4 | 18.5 ± 5.1 | 6.3 |
| | Control | 3 | 24.8 ± 6.0 | |
| gative | K46 | 3 | 29.7 ± 6.4 | 1.2 |
| | Control | 4 | 28.5 ± 1.6 | |

Table V.

| Donor (group) | No. of mice | Reticulocytes per. mille | | | Mean diff. ± s.e. (4th day - 0 day) |
|---|---|---|---|---|---|
| | | 0 | 4 | 10 day | |
| K26 (dbf.) | 6 | 22.5 | 26.1 | 28.9 | 3.6 ± 2.7 |
| K38 (dbf.) | 5 | 20.9 | 27.0 | | 6.1 ± 3.8 |
| K44 (pos.) | 6 | 15.7 | 72.9 | 47.2 | 56.1 ± 22.0** |
| K48 (neg.) | 6 | 27.8 | 16.7 | 26.3 | −11.0 ± 3.4** |

Reticulocyte response in intact mice after one injection of plasma from 4 stimulated rabbits.

viations from the mean of a sample occur with all methods in general use and explain the low p values in series with great differences between the means of the stimulated and control group. Lowy *et al.* (1959) discarded the highest reticulocyte value in each group. However, as the greatest deviations occur with plasma giving positive responses, in the control groups the deviations, at least in $Fe^{59}$ per cent uptake, are consistently smaller, this procedure may give misleading negative results.

More than one method has been used to test the content of erythropoietic stimulating factors in the plasmas from the stimulated rabbits in order to increase the reliability of the observations. Statistical methods can, however, hardly be applied to compare the results arrived at with these different methods. On the other hand, the rise both in reticulocytes and $Fe^{59}$ per cent uptake in the recipient animals strongly suggests that the donor plasma contains increased amounts of erythropoietins.

Plasma from K22, K28 and K44 showed significant increases both in $Fe^{59}$ per cent uptake and reticulocytes, while plasma from K23, K24 and K 47 showed significant increases with one of the methods, and definit however not significant increases, with t other method. These findings must be tak as evidence that the plasmas from the s rabbits contained increased amounts erythropoietic stimulating factors followi the hypothalamic stimulation.

Besides starved mice, intact and pol cythemic mice have been used. Jacobson *al.* (1959) consider polycythemic mice t most reliable recipients to show the conte of specific erythropoietins. Osnes (1959), the other hand, defines erythropoietins

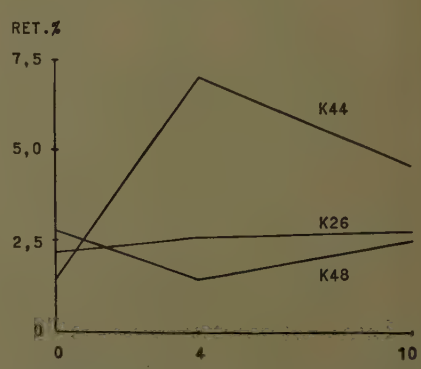


Fig. 2. Reticulocyte response in intact mice a one injection of plasma from 3 stimulated rabb See Table V.

Table VI.

| Donor | No. of mice | Reticulocytes per cent | | | Mean diff. ± s.e. (4th day - 0 day) |
|---|---|---|---|---|---|
| | | 0 | 4 | 10 day | |
| Control | 4 | 0.08 | 0.03 | 0.04 | −0.038 ± 0.014 |
| K44 | 6 | 0.04 | 0.37 | 0.02 | 0.327 ± 0.273 |

Reticulocyte counts in polycythemic mice after one injection of plasma from K44 and a contnol rabbit plasma.

ubstances giving rise to increased erythro-oiesis in intact recipients. These methods ave therefore been used to control the ndings pertaining to starved recipients and o test the possibility of non-specific re-ctions.

Plasma from 4 rabbits have been tested in ntact mice. K44 (pos.) caused a significant ise in reticulocytes 4 days after the injection o the same extent as a very active plasma Figs 1 and 2). K26 and K38 (dbf.) both nowed slight increases while K48 (neg.) aused a drop in the reticulocyte levels. Only K44 and a normal rabbit plasma have been ested in polycythemic mice, and K44 in-reased the reticulocytes in these recipients oo, however not significantly.

The findings of increased erythropoietic ctivity in starved, polycythemic and intact nice after injection of plasma from one abbit in the positive group, indicate that ne response in starved mice is not due to on-specific substances. Although only one f the positive plasmas has been tested in ntact and polycythemic mice, it is likely that ne mechanism has been the same in the ther 5 plasmas causing increased erythro-oiesis in starved mice.

It can be concluded that plasma from 6 of the stimulated rabbits showed evidence of increased erythropoietin levels. Table III shows the highest reticulocyte value during the stimulation period and the reticulocyte value at the time when the blood was withdrawn from the rabbits. In al 6 these values are the same or approximately the same, showing that the stimulus for erythropoiesis was maximal. In K35 (pos.) which showed no evidence of increased amounts of erythropoietins in the plasma, the reticulocytes had fallen from 63 per mille to 38 per mille at the time of blood sampling. On the other hand, K28 (dbf.) only showed reticulocytosis during the last 3 to 4 days of the stimulation period, and this may explain the slight increase in RCM (3.6 ml) in this rabbit, which is why this rabbit has been placed in the doubtful response group.

The findings presented in this paper favor the hypothesis that the increased erythrocyte formation in the stimulated rabbits has been due to humoral factors and not to a direct nervous influence on the bone marrow. Whether these humoral factors are the same as the erythropoietins occurring in anoxemic conditions cannot be stated, but it seems pro-

bable as they increased erythropoiesis both in starved, intact and possibly in polycythemic mice.

An important question is how stimulation of the hypothalamus may influence production of the erythropoietic stimulating factor(s). One possibility is the existence of a hypothalamic area controlling erythropoietin production. The factor(s) might either be produced in this area or, the area in question may through nervous (or humoral?) influence to a target organ (kidney?, liver?), stimulate erythropoietin production. The latter theory has some experimental basis in the findings of Heilmeyer (1933); *i. e.* that cross-section of the spinal cord in the cervical spine abolishes the erythropoietic effect of bleeding. In the present study no attempt has been made to investigate these possibilities. The possibility that increased ACTH production following hypothalamic stimulation may be the cause of the increased erythrocyte formation will be discussed in another publication.

## SUMMARY

We have previously reported that electric stimulation of the hypothalamic region in rabbits may induce reticulocytosis and an increase in red cell mass. In the present paper evidence is presented indicating that plasma from the stimulated rabbits showing a positive erythropoietic response, contains an increased amount of substances (erythropoietins) which stimulate erythropoiesis in starved, intact and polycythemic mice. These findings favor the concept that the effect of hypothalamic stimulation on the erythropoiesis is mediated through a humoral mechanism, and that the erythropoietin productio is subject to cerebral regulation.

## ACKNOWLEDGMENTS

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# SERUM HAPTOGLOBIN IN INFANCY

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Haptoglobin (Hp) is defined as a group of closely related mucoproteins with the electrophoretic properties of an $\alpha_2$-globulin, forming with hemoglobin a very stable complex (HbHp). The serum haptoglobin level in a given healthy individual is relatively constant (Jayle & Boussier, 1955; Nyman, 1959) but the normal values vary within wide ranges, 30 to 200 mg per 100 ml serum. It is well known that the haptoglobin level is changed during certain pathological conditions (*cf.* Nyman, 1959). It is *e. g.* increased in patients with acute infections and decreased when intra- or extravascular hemolysis is present.

It is usually stated (Lacomme cit. Polonovski, 1947; Tuttle, 1955; Wieme, 1956; Nyman, 1959; Bocci, Castelli & Cordelli, 1959; Weippl, 1960) that haptoglobin is rarely demonstrated in newborns but there seems to be no systematic investigation published concerning the haptoglobin levels in infancy. According to Nyman (1959) the haptoglobin seems to rise fairly rapidly in full-term infants.

The purpose of the present investigation was to study in more detail the haptoglobin levels during infancy.

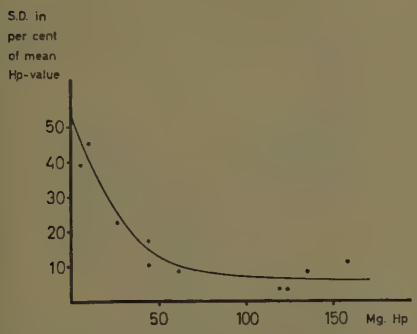
## MATERIAL AND METHODS

Blood was taken by heart puncture from 6 fetuse ranging in length from 16 to 26 cm. Samples c cord blood were collected at 29 normal deliverie with the birth weight of the newborns varyin between 2430 and 4380 g. In the rest of the new borns and in the infants, blood samples were ob tained by puncture of the sole. All infants wer healthy judging from routine clinical examinatio or had been admitted to hospital for minor disorder of non-infectious nature.

Haptoglobin was determined according to th activation method of Jayle as described by Nyma (1959). The method is based on the peroxidas properties of the HbHp-complex. The activity determined in a system of ethyl hydroperoxide an potassium iodide. Horse hemoglobin was used i the present investigation.

According to Nyman (1959) the reproducibilit of the method is good (average S. D. 3.8 mg/10 ml) but varies with the amount of haptoglob present in the sample. The error of the metho was determined in the present investigation b repeated analyses of 10 serum samples chosen random. As seen from the diagram (Fig. 1) th error increases considerably when only sma amounts of haptoglobin are present.

In addition, a paper electrophoretic method deve oped by Tarukoski was used. This method, originally carried out, proved, however, to ha considerable sources of error when applied samples with small amounts of haptoglobin. Th method has thus only a limited interest for t




g. 1. Standard deviation in per cent of mean ptoglobin values (average of 6 determinations).

esent investigation and will only be briefly de-ribed. Serum is mixed with a known amount of moglobin in excess of the hemoglobin-binding pacity of the serum. The HbHp-complex is sep-ated from the free hemoglobin by paper electro-oresis at pH 6.9 and stained with o-dianisidine. ie quantitative evaluation is made by a Spinco nalytrol or similar instrument. It is thus possible calculate the amount of haptoglobin from the lues for free and bound hemoglobin as the total nount of hemoglobin is known.

Bilirubin was determined according to the meth- of Jendrassik & Gróf as modified by Nosslin 960).

## RESULTS

All figures concerning haptoglobin values fer, when not otherwise stated, to the tivation method of Jayle.

The peroxidase activity of human fetal bHp-complex was determined by use of a rified human haptoglobin preparation and mpared with the activity of horse hemo-bin complex and of adult human hemo-bin complex. It was found that the fetal bHp-complex had an activity somewhat ver than horse hemoglobin complex and her than the adult hemoglobin complex ig. 2).

*Fetal sera.* In 6 different samples no haptoglobin was found.

*Cord blood.* No haptoglobin could be demonstrated with the method of Jayle in 29 samples of blood taken from the umbilical cord at delivery. With the paper electrophoretic method of Tarukoski a low peroxidase activity, apparently not due to free hemoglobin, was found in most samples.

*Newborn prematures.* 24 samples were taken from 18 newborns 1 to 11 days old with a birthweight ranging from 1370 to 2440 g. In 15 samples no haptoglobin could be demonstrated and in 8 samples a very slight peroxidase activity was found corresponding to values for the hemoglobin-binding capacity, which were below or in the vicinity of the sensitivity of the method. In one infant a small amount of haptoglobin (15 mg/100 ml) was demonstrated. The mother had hypertension and albuminuria.

With one exception (this infant had no visible jaundice) serum bilirubin was determined on the same day as haptoglobin. The

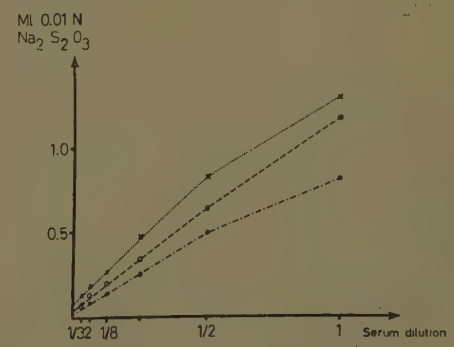



Fig. 2. Comparison between the peroxidase activity of human haptoglobin bound by horse hemoglobin and by fetal and adult human hemoglobin.
····· Hp horse Hb. ----- Hp fetal Hb.
—.—.— Hp adult Hb.

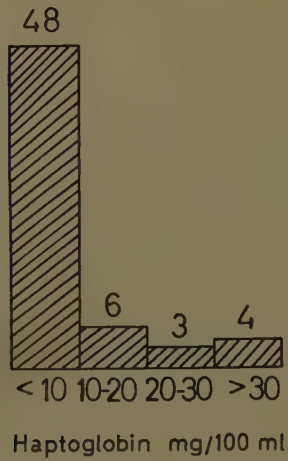



Fig. 3. Amount of haptoglobin in 61 serum samples from 55 fullterm newborn infants aged 1 to 12 days.

values ranged from 2.8 to 20.3 mg/100 ml. An examination of the blood groups of mother and infant was made in all of the prematures but in none could a serological explanation to the increased bilirubin level be demonstrated.

*Newborn full-term infants.* 61 samples were obtained from 55 infants ranging in age from 1 to 12 days. The samples were not quite evenly distributed over this period. The majority of the samples were collected during the first part of the newborn period, only 24 samples represented the 6th to the 12th day. As seen from the diagram (Fig 3), only four values exceeded 30 mg/100 ml, which is usually considered as the normal lower limit for adults. The maximum value obtained was 50 mg/100 ml. Of the samples with a haptoglobin content over 10 mg/100 ml, 5 were collected before the fourth day of life. The haptoglobin levels did not show a tendency to increase at the end of the newborn period.

Serum bilirubin was determined in samples. In the other sera no icteric co could be observed and the infants had visible jaundice. A complete serological e amination was made in most of the infa with high bilirubin values and in th mothers. In two cases maternal antibod were found with effect *in vitro* on the r cells of the infants. No haptoglobin could demonstrated in these cases.

The bilirubin values were plotted agai the haptoglobin values as shown in the d gram (Fig. 4). A very low haptoglobin c tent, less than 10 mg/100 ml, was found be combined with high as well as low b rubin values. Infants with demonstra levels of haptoglobin, more than 10 m 100 ml, showed with one exception, 1 serum bilirubin values.

*Older infants.* Samples from 92 full-te infants, 14 days to one year old, were a lysed. Of the infants 36 were 6 months older. The results are given in the diagra (Figs. 5 and 6). The haptoglobin val

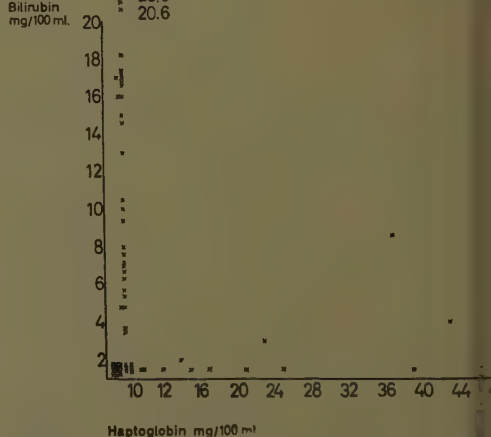
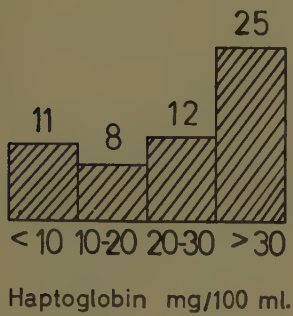


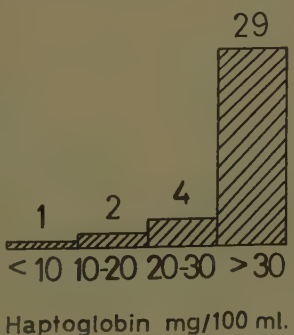

Fig. 4. Haptoglobin values correlated to bili levels in fullterm newborn infants aged 1 to 12 d




ig. 5. Amount of serum haptoglobin in 56 infants aged 14 days to 5 months.

aried within wide ranges in all age groups ut showed a tendency to increase with age Table I). During the first few months of fe "subnormal" values were the rule. After months haptoglobin levels below 30 mg/ 00 ml serum were rarely encountered. Only ne infant (aged 8 months) showed a value ver 200 mg/100 ml.

The haptoglobin values were also deter- ined in 12 prematurely born infants with n age between 19 days and 9 months. The esults were in good agreement with those btained in the full-term infants but as the umber of observations is too small, no de- nite conclusions can be drawn.




g. 6. Amount of serum haptoglobin in 36 infants aged 6 to 12 months.

## DISCUSSION

The present investigation confirmed the result of previous studies of the serum haptoglobin level in newborns. As a rule significant amounts of haptoglobin cannot be demonstrated in newborns with present methods. The cause of the low or absent peroxidase activity during this period of life is difficult to establish but some points ought to be taken into consideration.

Nyman (1959) has discussed definite and possible sources of error of Jayle's method and emphasized the influence of sampling hemolysis. The peroxidase activity of the Hp-complex with human hemoglobin is only about half of that of the horse hemoglobin complex. Analysis of hemolytic sera will thus give too low haptoglobin values when horse hemoglobin is used as the haptoglobin will partly or completely be bound to the human hemoglobin released. Hemolysis, not detected by inspection of the serum, has according to Nyman (1959) only a minor effect on the activity. In order to test the influence of hemolysis, the activity of serum samples with a known haptoglobin content and with different degrees of hemolysis was determined. It was found that even small amounts of adult

Table I. *Haptoglobin valus expressed in mg per 100 ml serum for various age groups.*

| Age group | Number of cases | Observed range | Median value |
|---|---|---|---|
| 14d – 2 mo | 26 | 0 – 78 | 19.5 |
| 3 & 4 mo | 21 | 0 – 80 | 45 |
| 5 & 6 mo | 16 | 0 – 145 | 44 |
| 7 & 8 mo | 17 | 19 – 183 (284) | 66 |
| 9 –12 mo | 12 | 23 – 198 | 92 |

human hemoglobin not visually detectable decreased the activity appreciable. There are also two other factors which must be taken into consideration. The majority of the newborns of the present investigation had bilirubinemia and it is well known that the hemoglobin content of icteric sera is easily underestimated. The matter is also complicated by the fact that fetal hemoglobin, which must at least be a part of the hemoglobin released by hemolysis in newborns, seems, bound to haptoglobin, to have a higher activity than the adult human hemoglobin-haptoglobin complex. It is thus possible that sampling hemolysis of a moderate degree will be of somewhat less importance in sera from newborn infants. As it is difficult entirely to avoid hemolysis when blood samples are taken from infants with the method used, it cannot be wholly excluded that this error has influenced the results. It seems, however, very unlikely that the failure to demonstrate haptoglobin in the majority of the newborn infants should be due to sampling hemolysis.

With the paper electrophoretic method of Tarukoski, peroxidase activity could be demonstrated in sera which according to the method of Jayle should be free of haptoglobin. This discrepancy is most probably due to heme-binding proteins which have no activity in Jayle's system (Nyman, 1959) and the findings may not be taken as proof that haptoglobin was present.

In this connection the relation of the haptoglobin level to the degree of bilirubinemia must be mentioned. It is well known that in cases of anemia with increased destruction of the red cells, haptoglobin usually is absent or found in subnormal amounts. According to modern conception, bilirubi emia in the newborn is caused by a deficie bilirubin glucuronide formation and not increased erythrocyte destruction. This do of course, not exclude that in newbor showing blood group incompatibility wi their mothers hemolysis may play an i portant role for the degree of hyperbilirubi emia. In cases of this type, not represent in the present investigation, a failure to d monstrate haptoglobin can be explained due to hemolysis. In newborns without ser logical incompatibility, measurable levels haptoglobin could perhaps be expected, no *abnormal* erythrocyte destruction is pr sent. This is not the case judging from t data presented here. In full-term newbor as well as in prematures without signs blood group incompatibility, absence of ha toglobin was observed regardless of low high bilirubin levels. In newborns, especial in prematures, the life span of the erythr cytes is, however, normally somewh shorter than in older infants and childr (*cf.* West, 1959) and this may possibly co tribute to the low or not measurable hapt globin levels in newborns even if it is n the cause of the "physiological jaundice".

Preliminary experiments to inject a pu fied haptoglobin solution into newbor suggest that the substance is rapidly elim nated from the circulation. When 30— mg haptoglobin was given intravenous measurable quantities could be demonstrat after a few minutes but not 4 to 5 hours lat No conclusions can be drawn but it tempting to assume that the injected hap globin was bound to hemoglobin, as it been shown (Laurell & Nyman, 19 Allison & Rees, 1957) that injected hem

lobin is bound to haptoglobin and the com-lex eliminated.

There are, however, other factors which ıust be taken into consideration. The poss-ıility that fetal hemoglobin is not bound to aptoglobin to the same extent as adult emoglobin has been excluded by van Royen 1950), nor does it agree with the present inding of a high peroxidase activity in a ystem with fetal hemoglobin. The presence f substances in serum from newborns in-ibiting the peroxidase activity of the HbHp-omplex cannot be excluded without further ıvestigations, even if it *apriori* does not eem very likely that such substances should lay any major role.

The most attractive explanation of the bsent or very low peroxidase activity found ı fetal and newborn sera with the method f Jayle is no doubt an inability of the fetal nd neonatal organism to produce hapto-lobin. The relatively low amount of $\alpha_2$-lobulins found in the fetus (Bergstrand & Czar, 1957) could possibly be in favor of ıis conception. It has, on the other hand, een demonstrated in the newborn (Ober-ıan, Gregory, Burke, Ross & Rice, 1956; osephson & Gyllenswärd, 1957) that the elative as well as the absolute amounts of $_2$-globulins differ only little from adult evels.

It is possible that the anhaptoglobinemia r low haptoglobin levels found in newborns re caused by several contributing factors iscussed above. Further investigations are ecessary to clarify this point.

It would have been of interest to follow a ufficient number of healthy infants for at ast the first six months of life but this was r different reasons not possible. Judging from the available data there is, after the newborn period, not an abrupt but gradual rise in the haptoglobin level. After 6 months "subnormal values" are rarely encountered. As in adults, normal values in infancy seem to vary within wide ranges but the upper limit appears to be the same. Determination of serum haptoglobin before 6 months of age seems to have a rather limited clinical value.

## SUMMARY

The serum haptoglobin level during fetal life and in infancy was studied by the activation method of Jayle as described by Nyman (1959).

Haptoglobin could not be demonstrated in fetal or umbilical cord blood.

Newborn premature infants did, as a rule, not show measurable amounts of haptoglobin.

In the majority of the newborn full-term infants, irrespective of the degree of jaundice, measurable quantities of haptoglobin could not be demonstrated. A few cases in this age group had haptoglobin levels exceeding 30 mg per 100 ml serum *i. e.* the lower limit for normal adults.

The haptoglobin levels tend to increase gradually during the first months of life and after 6 months relatively few infants show levels below 30 mg per 100 ml serum.

Possible causes of the anhaptoglobinemia during the newborn period is discussed and it is suggested that the most likely explanation is an inability of the neonatal organism to produce haptoglobin. Definite conclusions can, however, not be drawn from the present investigation.

Fetal hemoglobin bound to haptoglobin

appears to have a peroxidase activity which is higher than the corresponding complex of adult hemoglobin.

Preliminary experiments suggest that purified human haptoglobin injected intravenously into newborn infants is rapidly eliminated.

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# THE EFFECT OF ANEMIA ON BONE MARROW DEHYDROGENASE ACTIVITY AS TESTED BY THE REDUCTION OF 2,3,5-TRIPHENYLTETRAZOLIUM CHLORIDE

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(Received for publication March 1, 1961)

In a previous paper (Myren, 1960) it was ressed that a colorimetric determination f bone marrow dehydrogenase activity by ieans of 2, 3, 5 — triphenyltetrazolium hloride (TTC) may provide information on ie degree and type of anemia present. Thus, ie observation was made that in patients ith anemia caused by severe renal in-fficiency and metastasing carcinoma, the ctivity was lower than normal. On the other and, in patients with hypochromic anemia aused by bleeding or iron deficiency, and cases of pernicious anemia, the dehydro-enase activity usually was elevated. But the nall series of patients examined did not efinitely answer the following questions: ow is the bone marrow dehydrogenase tivity influenced by the degree of anemia tested by the concentration of hemoglobin? the bone marrow dehydrogenase activity patients with hypochromic anemia differ-t from that in cases with pernicious emia?

The answers to these questions may be of some importance for the diagnosis and prognosis. Therefore the present paper is concerned with the correlation of bone marrow dehydrogenase activity to the degree and type of anemia present.

## MATERIAL AND METHODS

Bone marrow specimens were obtained by puncture of the sternal corpus in local anesthesia. For morphological study, smears were stained by the method of Giemsa. The preparations were examined by routine microscopy. For dehydrogenase determination the method reported previously (Myren 1960, Lund-Roland & Myren 1961) was used. Bone marrow particles were incubated with a solution of two-thirds of 1 per cent TTC and one third of 0.1 M sodium succinate for one hour at 37° C. The red-colored formazan crystals, which were deposited in the cell cytoplasm proportional to the dehydrogenase activity, were then extracted with 5 ml of acetone for 24 hours. The color intensity was determined in a Beckman D.U. spectrophotometer, and the activity estimated in Beckman extinction values per mg dry weight. The weight of the dried marrow particles was from 0.3 to 10 mg, usually between 1 and 3 mg.

Table I. *The age of the patients.*

| Patients with | Age in yaers | | | | | Number of patients |
|---|---|---|---|---|---|---|
| | –40 | 41–50 | 51–60 | 61–70 | 71– | |
| pernicious anemia | | | | | | |
| females | | | | 1 | 5 | 6 |
| males | | 1 | | 2 | 5 | 8 |
| sideropenic anemia | | | | | | |
| females | | 2 | 2 | 5 | 1 | 10 |
| males | 1 | | 3 | 4 | 1 | 9 |
| chronic nephritis | | | | | | |
| females | | | 1 | | 1 | 2 |
| males | | | 1 | | | 1 |
| other diseases | | | | | | |
| females | 3 | | 2 | 1 | 6 | 12 |
| males | 3 | | 5 | 3 | 4 | 15 |
| Total | 7 | 3 | 14 | 16 | 23 | 63 |

The total material examined consisted of 63 patients. Of these, 33 were women and 30 were men (Table I).

Six women and 8 men had pernicious anemia. The diagnosis was supported by the typical megaloblastic morphological picture of bone marrow smears, by the result of Schilling's test and by the therapeutic response to the administration of $B_{12}$.

Ten women and 9 men had hypochromic anemia caused by iron deficiency or bleeding. An increased erythropoiesis was usually found in the bone marrow smears.

Two women and one man had chronic nephritis with a morphologically normal bone marrow.

Twenty-seven patients (12 women and 15 men) had different other diseases, some of which were accompanied by anemia (Table II). In 13 of these cases the concentration of hemoglobin was 13 g per cent or more. In 5 patients the concentration was 12 to 13 g per cent, and in 7 cases the concentration of hemoglobin was less than 12 g per cent. In bone marrow smears a normal morphological picture was found or an increase of the number of plasma cells. None of these patients had leukemia or aplasia of bone marrow.

## RESULTS

As shown in Fig. 1, a correlation w found between the dehydrogenase activity bone marrow and the concentration of hem globin of peripheral blood. Thus, the avera values of dehydrogenase activity increas significantly when the concentration of hem globin decreased from 14 to 11 g per ce (p-value between 1 and 0.5 per cent). similar increase of activity was found wh the concentration of hemoglobin was reduc from 11 to between 8 and 9 g per cent ( value between 1 and 0.5 per cent). Wh the Hb-concentration decreased still mo the degree of dehydrogenase activity i creased abruptly from 0.14 to 0.29. Beckm units (extinction values), with standa deviation 0.026 and 0.04 respectively. T difference of activity between these t groups of patients examined, was statistica highly significant (p-value $< 0.5$ per ce t-value 5.5). In this correlation patients wi chronic nephritis were not included.

The correlation of bone marrow dehyd genase activity to the diagnoses of diseas shows also that the concentration of hem

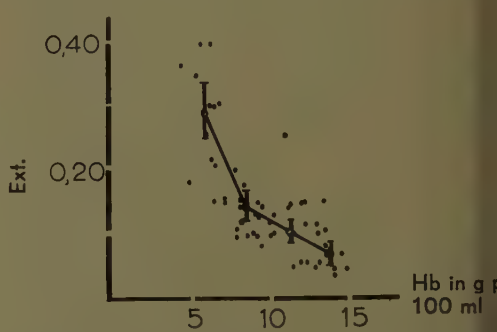
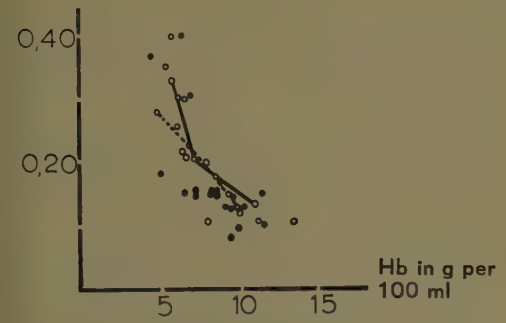



Fig. 1. Correlation between concentration of he globin in peripheral blood (Hb in g per cent) bone marrow dehydrogenase activity (Beckm units, extinction values), 60 patients.




g. 2. Comparison between bone marrow dehydro-nase activity in 14 patients with pernicious ane-ia (small circles) and the activity in 19 patients with sideropenic anemia (dots).

obin is of importance for the degree of de-ydrogenase activity (Table II and Fig. 2). n the patients with chronic nephritis, how-ver, a low concentration of hemoglobin was ccompanied by a low degree of bone marrow ehydrogenase activity.

Thus, in the group of patients with per-icious anemia the average value of dehydro-enase activity was 0.22 Beckman units, tandard deviation ± 0.05. The average alue of hemoglobin concentration in the eripheral blood of this group of cases was 8 g per cent with a standard deviation of ± 1.2.

In the group of patients with sideropenic nemia, the concentration of hemoglobin was 5 g per cent with a standard deviation of ± 0.9. In these cases the average value of ehydrogenase activity was 0.18 Beckman nits, standard deviation ± 0.05.

The bone marrow dehydrogenase activity pernicious anemia was significantly differ-nt from that found in patients with side-penic anemia as also demonstrated in ig. 2. But the values of activity in per-cious anemia and in sideropenic anemia cases were significantly higher than those found in patients with other diseases (Fig. 2 compared to Fig. 1, and Table II compared to Table III). In this group of cases, chronic nephritis excluded, the average dehydrogenase activity was 0.09 Beckman units with a standard deviation of ± 0.02. As might be predicted from Fig. 1, the concentration of hemoglobin was 12.1 g per cent, the standard deviation ± 1.1. Also in the group of patients with anemia associated with other diseases (pernicious anemia and sideropenic anemia excluded), the degree of dehydrogenase activity was dependent on the concentration of hemoglobin of peripheral blood. Thus, as shown in Table III, the dehydrogenase activity was between 0.06 and 0.07 Beckman units when the concentration of hemoglobin was normal or nearly normal. On the other hand, when the concentration of hemoglobin was lower than 12.5 g per cent, the dehydrogenase activity also was higher than normal. But, this elevation of dehydrogenase activity may be influenced in different ways by the diseases which were found together with anemia in these patients (Table III). Thus,

Table II. *Bone marrow dehydrogenase activity in relation to groups of diseases and concentration of hemoglobin. 63 patients.*

| Disease | No. of patients | Dehydrogenase activity. Beckman extinction val. | SD | Conc. of Hb in g per cent | SD |
|---|---|---|---|---|---|
| Chronic nephritis | 3 | 0.05 | 0.04 | 7.2 | 2.1 |
| Pernicious anemia | 14 | 0.22 | 0.05 | 7.8 | 1.2 |
| Sideropenic anemia | 19 | 0.18 | 0.05 | 8.5 | 0.9 |
| Other diseases | 27 | 0.09 | 0.02 | 12.1 | 1.1 |

Table III. *Bone marrow dehydrogenase activity in relation to diagnoses and concentration of hemoglobin in the group of patients with other diseases.*

| Diagnoses | No. of patients with average values of dehydrogenase activity (Beckman extinction values) | | | | | Conc. of Hb in g per cent |
|---|---|---|---|---|---|---|
| | 0.06–0.07 | 0,08–0.09 | 0.10–0.11 | 0.12–0.13 | 0.14– | |
| Temporal arteritis | | 2 | | | | 9.7 |
| Anemia of uncertain etiology | | | 7 | | | 10.5 |
| Pulmonary infections | | | | 5 | | 11.1 |
| Chron. rheum. arthritis | | 3 | | | | 11.3 |
| Osteoporosis | | | | | 1 | 12.1 |
| Thrombocytopenia | 2 | | | | | 12.5 |
| Disease of stomach | 3 | | | | | 13.0 |
| Disease of thyroid (non toxic) | 2 | | | | | 13.6 |
| Observation | 2 | | | | | 14.1 |

pulmonary infections and osteoporosis may produce an increase of activity in addition to that of anemia. On the other hand, chronic nephritis may depress the bone marrow dehydrogenase activity severely as shown in Table II. The average value of activity in patients with chronic nephritis was significantly lower than that found in the group of patients with other diseases irrespective of the lower concentration of hemoglobin in the small group of cases with nephritis (p-value $< 0.5$ per cent, $t = 2.7$).

## COMMENTS AND CONCLUSIONS

These results may give answer to the questions proposed.

Anemia usually causes an elevation of bone marrow dehydrogenase activity. This increase of activity is relatively slow when the concentration of hemoglobin is reduc from normal to about 8 g per cent as demo strated in Fig. 1. A further reduction of t hemoglobin concentration is associated wi an accelerated increase of dehydrogena activity. Thus, determination of bone ma row dehydrogenase activity may give info mation on the severity of anemia prese and of the effort of bone to compensate t blood deficit. Accordingly, an increas frequency of nuclear mitosis is usually fou in pernicious and sideropenic anemias. B different other diseases causing anemia m affect the degree of dehydrogenase activit In chronic nephritis with anemia the bo marrow dehydrogenase activity is lo whereas in acute infections the activity m be higher than expected (Table III).

The highest values of dehydrogenase acti ity were found in patients with pernicio and sideropenic anemia. This observati may show that a high value of dehydr genase activity is indicative of these blo diseases (Fig. 2 compared to Fig 3 a Table II). But the degree of dehydrogena activity in pernicious anemia seems not to significantly different from the bone marro activity found in sideropenic anemia (Fig.2

## SUMMARY

The role of anemia for the degree of bo marrow dehydrogenase activity has been e amined in 63 patients. Of these cases, 14 h pernicious anemia, 19 had sideropenic an mia, 3 had chronic nephritis with anemia a 27 patients had other diseases more or le affecting the hematopoesis (Table I, II, III

Bone marrow dehydrogenase activity creased significantly with reduction of t

ıcentration of hemoglobin in peripheral od. This elevation was most pronounced pernicious and sideropenic anemia (Fig. On the other hand, in chronic nephritis h anemia the bone marrow dehydro-ıase activity was lower than normal as o found previously (Normal values of ivity between 0.06 and 0.07 Beckman its (extinction values)).

Determination of bone marrow dehydro-ıase activity may thus give information on the severity of anemia present and of the functional effort of bone marrow.

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# DETERMINATION OF 2-AMINO-2-HYDROXYMETHYL-1-3-PROPANEDIOL (THAM)

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(Received for publication March 3, 1961)

2-amino-2-hydroxymethyl-1-3-propanediol, usually abbreviated THAM or TRIS, has recently been proposed as a suitable buffering substance for the management of acidosis (Nahas 1959). For the experimental investigations in this field the need arises to be able to assay this compound, in various body fluids, for example in urine and serum.

The following method is based on the oxidation of THAM with periodic acid and determination of the formaldehyde formed with chromotropic acid. Before the oxidation the compound is separated from interfering materials with the help of a cation exchange resin, Dowex 50.

## METHODS

*Reagents and apparatus* (All solutions prepared with analytical grade reagents.)

Ammonium chloride — 2M
Ammonium hydroxide — 0.05M
Periodic acid — 0.1M
Sodium bisulfite — 0.5M

Chromotropic acid reagent: 300 mg chromotropic acid, Na-salt, is dissolved in 25 ml water, and 75 ml conc. $H_2SO_4$ is added

Ion exchange column: Dowex 50W-X8, 1
200 mesh, is placed in a beaker and wash
repeatedly, first with 2M HCl, then 2M NaO
then water, the slowly sedimenting particles bei
decanted. 1 ml of the ion exchange resin is tra
ferred to a chromatographic column, 0.8 mm
120 mm.

The resin is converted to its $NH_4^+$-form w
10 ml 2M $NH_4Cl$ and is then washed with 20
water or until the wash water is chloride-fr
After use the resin is regenerated in the same w
with 2M $NH_4Cl$.

All measurements are made with a Beckm
Model B spectrophotometer in 1 cm glass cells.

*Procedure*

1 ml of sample, urine or serum, is used for
analysis if the THAM concentration does exc
150 mg/100 ml. If the concentration is higher
sample is diluted or a smaller amount taken.
sample is placed on the column and washed w
10 ml water. The THAM adsorbed on the re
eluted with 10 ml 0.05M $NH_4OH$ and the eluate
taken up in a 50 ml volumetric flask, which is fil
to the mark with 0.05M $NH_4OH$. 2 ml of
solution is transferred to a glass-stoppered
tube. 0.1 ml 0.1M periodic acid is first added,
5 minutes later 0.1 ml 0.5M sodium bisulfite
10 ml chromotropic acid reagent. The test tube
stoppered and placed in a boiling water bath
10 minutes. After cooling to room temperature

sorption is measured at 570 mμ. A blank test nsisting of 2 ml 0.05M $NH_4OH$ is run parallel the eluate.

A standard graph is prepared by assaying known ounts of THAM in 0.05M $NH_4OH$ omitting ion exchange step.

## DISCUSSION

A number of compounds containing an H-group adjacent to an OH-, $NH_2$- or O-group can give formaldehyde upon oxation with periodic acid (Mead & Bartron, 48, Jackson 1944). This reaction is thus t specific for THAM, and a large number compounds which react in this way are rmally found in both urine and serum. It is erefore first necessary to separate THAM om these compounds, which would give rge blank values. With the separation ethods here described it now becomes poss- e to eliminate these interfering substances. It has been found advantageous to use owex 50W-X8 in the ammonium form inead of the hydrogen form, one reason being at in the ammonium form none of the ine color is adsorbed. 1 ml of urine or rum containing no more than 1.5 mg HAM is eluted quantitatively with 10 ml 05M $NH_4OH$.

With increased ammonia concentration in e eluting solution the blank increases. This eans that some of the interfering subnces are not quantitatively removed with ter because they are more strongly adrbed than THAM to the ion exchange sin. Upon elution with 0.05, 0.1, and M $NH_4OH$, the same urine gave blank lues equivalent to $< 0.1$, 2.5, and 5 mg HAM/100 ml respectively.

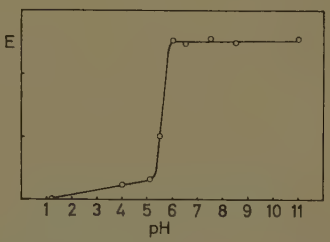


Fig. 1. Oxidation of THAM at varying pH. Oxidation time 5 min.

### *Oxidation of THAM with periodic acid*

As mentioned above compounds with adjacent OH- or $NH_2$-groups are oxidized by periodic acid (Mead & Bartron 1948). 1 mole THAM gives 2 moles of formaldehyde upon oxidation with periodic acid.

The oxidation is pH-dependent, and as can be seen from Fig. 1 it should be performed at a pH over 6.

The reaction with periodic acid seems to take place almost immediately. 53 mg THAM in 2 ml 0.05M ammonia is oxidized completely by 0.1 ml 0.1M periodic acid within one minute.

### *Determination of formaldehyde formed*

The color reaction which takes place with chromotropic acid in sulfuric acid has, in more or less modified forms, found a widespread use as a method of determination for formaldehyde (Bricker & Johnson 1945, MacFayden 1945), and it has even been used by a number of authors for the determination of formaldehyde formed after periodate oxidation (Frisell, Meech & Mackenzie 1954, Critchfield & Johnson 1957). As the reaction is performed here, the standard curve is a straight line within the tested range, which corresponds to an amount of 2-60 μg THAM, in the measured solution.

Concerning the color development, maximum color was obtained after 5 minutes warming time in the boiling water bath, and no difference in absorption could be observed. at times varying between 5 and 30 minutes.

The chromotropic acid reagent should have a faint yellow color. A strong brown-yellow color indicates that the chromotropic acid is impure. It can be purified by re-chystallization from a water-alcohol solution, from which the impurity precipitates first. The prepared reagent should not be stored for a very long time.

The color obtained with formaldehyde is stable for at least 18 hours.

*Precision of method*

The method has been tested for urine and serum with addition of THAM within the range 2.5 mg —1500 mg/100 ml, and has also been tested on pure water solutions, excluding the ion-exchange step. The results with these three different test media showed no significant difference. The average standard deviation ($\sigma$), within the test concentration range, was 0.34 $\mu$g for water solution, 0.58 $\mu$g for urine and 0.49 $\mu$g for serum.

## SUMMARY

A method for the determination of THAM in serum and urine has been elaborated. THAM is separated from interfering substances with a cation exchange resin, Dowex 50W-X8, and after oxidation with periodic acid the formaldehyde formed is determined with chromotropic acid.

## ACKNOWLEDGEMENT

The authors are indebted to tekn. lic. Ad Berggren for valuable discussions.

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## ADDENDUM

Since this paper was accepted for publication following two papers concerning the same subj have appeared:

Linn, S. & Roberts, M.: Microassay of Tr (hydroxymethyl) aminomethane applicable blood and urine. Ann. New York Acad. *92,* 419, 1961.

Rosen, H.: Quantitative measurement of the am buffer: 2-amino-2-hydroxymethyl-1,3-propa iol. Ann. New York Acad. Sci. *92,* 414, 1

andinav. J. Clin. & Lab. Investigation 591—593, 13, 1961.

# PURIFICATION OF HUMAN THROMBIN ON A CARBOXYMETHYL-CELLULOSE COLUMN

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(Received for publication March 17, 1961)

Strøier Rasmussen's method (1955) for rification of bovine thrombin on a column the cation exchange resin Amberlite IRC- has successfylly been applied in this boratory. We have found it difficult, how- er, to use the same method for the puri- ation of human thrombin. The thrombin as found to be hard to elute from this lumn and the yield was low. The method scribed below is easy and rapid to perform, d gives a good yield of a human thrombin high purity.

## MATERIALS AND METHODS

*Accelerin* was prepared by chromatography on anion exchange resin column, (Korsan-Bengtsen Ygge, 1961).

*Carboxymethyl-cellulose column.* Carboxymethyl-lulose (CM 70 from Whatman, delivered by H. eve Angel & Co. Ltd., London) was suspended physiological saline containing 1/5 its volume a 0.15 M phosphate buffer, pH = 6, and was owed to sediment slowly through a funnel into glass column which had been filled with the xture of physiological saline and 0.15 M phos- te buffer, pH = 6 until it formed a column of boxymethyl-cellulose about 20 x 200 mm.

*Conductivity measurements* were performed with Philips conductivity measuring bridge, GM 4249/01, with the measuring cell GM 4221 at room temperature. 0.2 ml of the fractions to be measured was diluted with 15 ml of redistilled water and the resistance was determined in these solutions.

*Crude thrombin.* 200 ml of human oxalated plasma was adsorbed with $BaSO_4$ (100 mg/ml). The $BaSO_4$ was washed 3 times with physiological saline by suspension and centrifugation, and the $BaSO_4$ was eluted with 20 ml of a 6 per cent trisodium-citrate solution. 2 ml of the tissue thromboplastin preparation described below, 2 ml accelerin, and 4 ml of $CaCl_2$ 1 M were added. The mixture was dialyzed against running tap-water for 48 hours and then centrifuged in an "International" refrigerated centrifuge at 19 000 R.C.F. for 60 minutes. It was divided into portions of 5 ml and stored at —20° C. It was not necessary to add accelerin. The thrombin yield, however, was somewhat higher in the preparations in which accelerin was present.

This crude thrombin contained 12-20 thrombin units (see below) per mg protein.

*Phosphate buffers*

pH = 6.0 (8.8 ml of 0.15 M $KH_2PO_4$ + 1.2 ml of 0.15 M $Na_2HPO_4$).

pH = 8.0 (0.5 ml of 0.5 M $KH_2PO_4$ + 9.5 ml of 0.5 M $Na_2HPO_4$).

*Protein determination methods.* The protein concentration in the crude thrombin was calculated from the nitrogen concentration determined by Kjeldahl procedure. Multiplication factor = 6.25.

The method of Lowry, Rosebrough, Lewis Farr

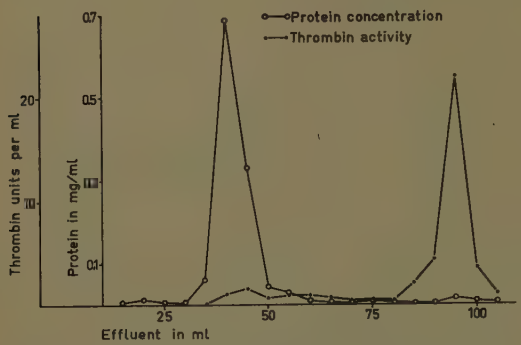


Fig. 1. Separation of "thrombin" and inactive proteins on the carboxymethylcellulose column.

& Randall (1951) with the Folin-Ciocalteu reagent was used for the determination of protein in the fractions. Dilutions of the crude thrombin served as standards.

*Sephadex 25* from Pharmacia, Uppsala, Sweden. The columns were made as described above by suspending the Sephadex in water and letting it sediment into a 20 x 400 mm glass column. The column of Sephadex used was 20 x 100 mm.

*Thrombin activity determination method.* 0.2 ml of the thrombin solution of various dilutions was blown down into 0.2 ml human fibrinogen, 0.2 per cent, dissolved in physiological saline containing 1/5 its volume of isotonic veronal buffer, pH = 7.3, (Owren 1947), and the clotting time recorded in a water bath of 37° C. The dilution which gave a clotting time of 15 seconds was said to contain 2 thrombin units per ml. 1 thrombin unit (T.U.) thus is able to clot 1 ml 0.1 per cent fibrinogen in 15 seconds under the circumstances mentioned. This is the definition of 1 thrombin unit given by Owren (1947). When compared to the commercial bovine thrombin preparation "Topostasin Roche", this thrombin unit seems to correspond to about 3 N.I.H. units.

*Tissue thromboplastin.* Human brain thromboplastin prepared as described by Owren (1947) was centrifuged at 19 000 R.C.F. The sediment was resuspended in physiological saline and again centrifuged. This washing was repeated 3 times after which the suspension was used.

## EXPERIMENTS AND RESULTS

1. *Separation on the carboxymethyl-cellulose column.* 5 ml of the crude thromb was placed on the carboxymethyl-cellulo column and allowed to drain in. 50 ml of mixture of 4 volumes of physiological sali and 1 volume of 0.15 M phosphate buff pH = 6 were run through. Then the eluti was performed with a solution containi 4 volumes of NaCl, 0.5 M, and 1 volume phosphate buffer, 0.5 M pH = 8. The elua was collected in portions of 5 ml and an lyzed for thrombin activity and protein co tent. Conductivity measurements were pe formed in the fractions and corrections to t conductivity of physiological saline we made by dilution in appropriate volumes distilled water before the determination thrombin activity. The result of an expe ment is schown in Fig. 1. There is, as w be seen, a good separation of the thromb activity from inactive proteins.

2. *Removal of the buffer and NaCl*

The fraction from the carboxymethyl-c lulose column which contained the highe activity was placed on a 2 x 100 mm colu

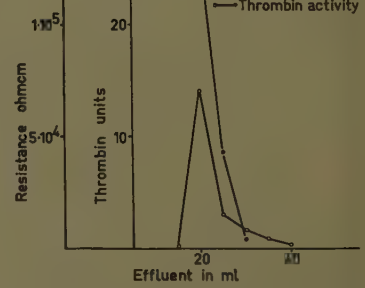


Fig. 2. Removal of the buffer and the NaCl on Sephadex-25 column.

Sephadex-25 which had been run through th distilled water. The fraction was owed to drain in, and the elution was per-rmed with distilled water. Fig. 2 shows at almost all of the thrombin activity ap-ared as a single peak after about 15 ml of ater had passed through the column. The nductivity in the fraction with the highest tivity was almost similar to that of distilled ater. This thrombin usually lost about 10 r cent of its activity when it was frozen at -20° C and about 50 per cent of its activity hen it was freeze-dried.

## SUMMARY

The separation of human thrombin from active proteins on a carboxymethyl-cellulose column and the subsequent removal of the buffer and NaCl on a Sephadex column are described.

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*Scandinav. J. Clin. & Lab. Investigation 594—603, 13, 1961.*

# MECHANISM OF THE CLOT-PROMOTING EFFECT OF FATTY ACIDS AND SYNTHETIC ANIONIC DETERGENT

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(Received for publication March 17, 1961)

In 1916, Stuber and Heim observed that certain fatty acids shorten the recalcification time of oxalated horse plasma. During recent years, some attention has again been paid to this problem because it was thought that the increased coagulability, which is observed after a fatty meal, might be caused by an increased concentration of free fatty acids in the blood.

Recent studies on the subject have been published by Poole (1955). She observed that sodium palmitate, sodium stearate, sodium oleate, and sodium laurate were able to shorten the clotting times of human plasma both with and without the presence of Russell's viper venom (R.V.V.). O'Brien 1957 could demonstrate this activity of the fatty acids only in the presence of R.V.V. Pilkington (1957), who worked with recalcification tests without the presence of R.V.V., found the unsaturated fatty acids oleic, linoleic, linolenic acids, and the saturated acids with 12 and 14 carbon atoms to be inactive. On the other hand, he found that the saturated acids with 16-20 carbon atoms were active.

The main purpose of the present work h been:

1. to investigate the importance of t length of the hydrocarbon chain of the satu ated fatty acids for their clot-promoting a tivity,

2. to determine whether unsaturated fat acids and some other anionic substances wi a hydrophobic tail promote the clotting,

3. to study some details concerning t mechanism by which these substances acc lerate the thrombin formation.

As it has been shown in previous wo (Korsan-Bengtsen, 1960) that fatty aci with a long hydrocarbon chain inactiva thrombin and prolong the thrombin-fibi nogen clotting time, the present study h been concentrated on their effect on t thrombin generation. For this study it w necessary to use special clotting systen The composition of these systems and t preparation of their components will be pi sented.

## MATERIALS AND METHODS

*Accelerin:* (Serum Ac-globulin) was prepared a modification of the method of Cox, Lanchan- & Ware (1955). The details of this method are scribed elsewhere in this journal (Korsan-Bengt- n & Ygge, 1961).

*Adsorbed plasma:* Human oxalated plasma, col- cted as described below, was adsorbed with $BaSO_4$ C.P. 100 mg/ml.

*Buffered physiological saline:* 4 volumes of phy- ological saline + 1 volume of the isotonic veronal ffer of Owren, pH = 7.3, (Owren 1947).

*Cholate:* Sodium cholate puriss from E. Merck G., Darmstadt, Germany.

*Clotting technique:* The tests were performed small thin-walled test tubes, 9 x 35 mm in size, hich were continuously tilted in a thermostat- gulated water bath at 37° C ± 1° C and observed r clotting. The time was measured with ordinary op watches.

*Collection of plasma:* Blood was collected from e antecubital vein with coarse and sharp silicone- eated needles into lusteroid tubes and mixed with 10 its volume of 3 per cent trisodium citrate or per cent dipotassium oxalate. Centrifugation was rformed in an "International" refrigerated cen- ifuge (model PR-2) at 1500 R.C.F. at 4° C for minutes.

*Dinonylsulfosuccinate:* The commercial com- und "Manoxol 100 % pure" from Hardman Hol- n Ltd., Manchester 10, England.

*Dodecylbenzenesulfonate:* The same substance hich was used in a previous paper (Korsan-Bengt- n 1960).

*Eluate:* 200 ml human oxalated plasma was ad- rbed with $BaSO_4$ (100 mg/ml). After washing e $BaSO_4$ three times with buffered physiological ine, the elution was performed with 20 ml 6 r cent trisodium citrate. The eluate was dialyzed e hour against running tap-water and then for o days at + 4° C against 4000 ml of distilled ter containing 0.05 per cent of trisodium citrate. e dialysis fluid was changed five times. After dialysis, the eluate was freeze-dried. The dry bstance was powdered and extracted three times with 100 ml portions of chloroform-methanol 3:1 at —20° C. The powdered eluate was allowed to be in contact with the chloroform-methanol solution for 30 minutes every time. After the last extraction the powder was taken directly to a vacuum ex- siccator and dried. It was kept in a stoppered tube at —20° C.

Before use, it was dissolved in a volume of buffered physiological saline corresponding to 1/10 the volume of the eluate before it was freeze-dried. The small quantity of the dried eluate which was insoluble, was removed by centrifugation. This final solution is henceforth referred to as "the eluate". It contained approximately 0.5 per cent trisodium citrate, and had a prothrombin activity which was about 50 times that of normal human plasma, tested with the prothrombin determination method of Hjort, Rapaport, and Owren (1955). In most of the experiments, dilutions of this "stock eluate" were used. The dilutions were made with buffered physiological saline containing 0.5 per cent trisodium citrate.

*Fatty acids:* The same substances which were used in a previous paper (Korsan-Bengtsen 1960).

*Fibrinogen:* Human fibrinogen prepared by Blombäck & Blombäck's method (1956) delivered by Kabi AB, Stockholm, Sweden. Before use, it was dialyzed against buffered physiological saline for 48 hours.

*Gangliosides:* The same substance which was used in a previous paper (Korsan-Bengtsen 1960).

*Lipid material:* Human brain, washed and ground, was treated 6 times with an excess of ace- tone. 1 g of the dry powder was extracted with 60 ml chloroform C.P. The chloroform was eva- porated, and the residue (300 mg) suspended in 50 ml of buffered physiological saline. It was kept in small test tubes at —20° C.

*Lysed platelets:* 90 ml of blood was collected in a lusteroid tube and mixed with 10 ml of a 2 per cent solution of disodium sequestrene (disodium ethylenediaminetetraacetate Merck AG., Darmstadt, Germany). After centrifugation for 10 minutes at 170 R.C.F. in an "International" refrigerated cen- trifuge (model PR-2), the platelet-rich plasma was collected and recentrifuged for 5 minutes. The

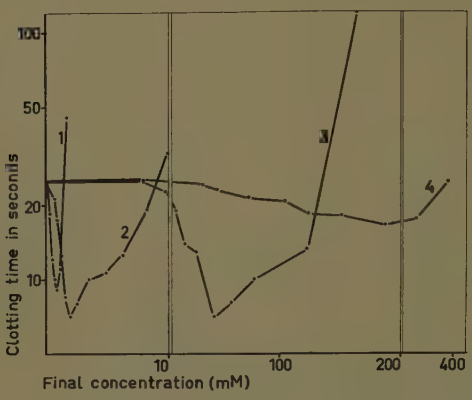



Fig. 1. Effect of sodium dodecylbenzenesulfonate, sodium laurate, sodium caprinate, and sodium caprylate on the clotting times of plasma in the presence of R.V.V.

Clotting system: 0.2 ml human citrated, platelet-poor plasma + 0.2 ml R.V.V. diluted 1/10000 with buffered physiological saline + 0.2 ml of the different fatty acids or sodium dodecylbenzenesulfonate of various concentrations. After 5 minutes incubation at 37° C, 0.2 ml $CaCl_2$ 0.025 M was blown down and the clotting time recorded.

Abscissa: final concentration of the different fatty acids and sodium dodecylbenzenesulfonate. Ordinate: logarithmic scale.

1 = sodium dodecylbenzenesulfonate.
2 = sodium laurate.
3 = sodium caprinate.
4 = sodium caprylate.

platelets were then sedimented at 1500 R.C.F. for 30 minutes, and washed 3 times in buffered physiological saline by resuspension and recentrifugation. Finally, they were suspended in 5 ml of buffered physiological saline and kept at —20° C until the day they were used. Before use the lysed platelets were heated to 60° C for 15 minutes, after which no proaccelerin activity was left in the solution.

*Proaccelerin* (fact. V) was prepared as described by Hjort (1957). The details of this method are described elsewhere in this journal (Korsan-Bengtsen & Ygge, 1961).

*Proaccelerin determination method:* A modification of the one-stage method of Aas (1952) was used. (Korsan-Bengtsen & Ygge, 1961).

*Russell's viper venom (R.V.V.):* Stypven, Burroughs Wellcome & Co., London, England.

*Thrombin:* Human thrombin prepared as described elsewhere in this journal (Korsan-Bengtsen & Ygge, 1961).

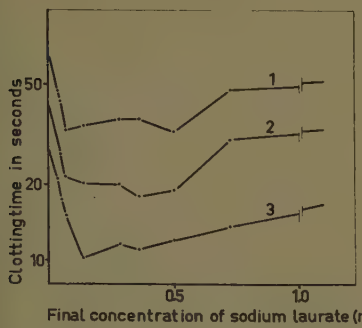
## EXPERIMENTS AND RESULTS

### *Effect of Fatty Acids with a Varying Length of the Hydrocarbon Chain and of Dodecylbenzenesulfonate on the Clotting of Plasma in the Presence of R.V.V.*

The experiments were performed by mixing 0.2 ml of human citrated, platelet-poor plasma with 0.2 ml R.V.V. (1/10000) and 0.2 ml of the different substances to be tested in various concentrations. After 5 minutes of incubation at 37° C, 0.2 ml $CaCl_2$ 0.025 M was blown down and the clotting times recorded.

The results are illustrated in Fig. 1. Rather low concentrations of sodium laurate and sodium dodecylbenzenesulfonate shorten the clotting times. Caprinate also shortens the clotting times to the same degree, but the concentration needed is about ten times that of laurate. Caprylate gives a slight decrease in the clotting times in very high concentrations.

It has also been shown in similar experiments that the sodium salts of the unsaturated fatty acids, oleic and linoleic acid, shorten the clotting times to the same degree in the same concentrations as laurate. The synthetic detergent dinonylsulfosuccinate behaves as dodecylbenzenesulfonate. Furthermore, sodium cholate and gangliosides have been tested and were also found to shorten the clotting times.




. 2. Clot-promoting effect of sodium laurate in
tting mixtures with a high concentration of pro-
ccelerin and different concentrations of eluate.
tting system: 0.1 ml eluate of different concen-
tions + 0.1 ml proaccelerin + 0.1 ml fibrinogen
% + 0.1 ml R.V.V. 1/10000 + 0.1 ml physio-
ical saline + 0.1 ml sodium laurate of different
centrations or physiological saline as a control
+ 0.1 ml $CaCl_2$ 0.04 M.
Ordinate: logarithmic scale.
eluate diluted 1:12 with buffered physiological
saline.
eluate diluted 1:5 with buffered physiological
saline.
eluate undiluted.

*bility of Sodium Laurate and Sodium decylbenzenesulfonate to Promote the tting in Clotting Systems Con- ning Lysed Platelets or Lipid terial from Brain.*

According to Rapaport, Aas & Owren 954), R.V.V. together with a lipid mate- has an activity in the blood coagulation ich is equivalent to convertin (SPCA). nen R.V.V. in a sufficient concentration is led to plasma, the thrombin generation, s depends upon the content in the plasma rothrombin (fact. II), proaccelerin (fact. Stuart factor (fact. X), lipid material, ium, and inhibitors. Consequently, an ease in the thrombin generation can be caused by an increased activity of one or several of these clot-promoting substances or by a decreased activity of inhibitors.

In the following experiments performed with clotting systems containing R.V.V. it will be shown that sodium laurate has no accelerating effect on the clotting when lysed platelets or lipid material from brain is present. This is the case both with low and high concentrations of eluate and proaccelerin in the clotting systems. However, without lysed platelets or lipid material present, an accelerating effect of sodium laurate is demonstrable at all tested concentrations of eluate and proaccelerin.

The first experiment is shown in Fig. 2. There was a high concentration of proaccelerin in the clotting system, and a varying concentration of the eluate obtained from human plasma as described in the section "Materials and methods". As will be seen, a high concentration of eluate did not eliminate the effect of the fatty acid. Quite the contrary, the fatty acid was more active when the concentration of eluate was high.

Fig. 3 shows an experiment with the same clotting mixture but an optimal concentration of lysed platelets had been added. There was no shortening of the clotting times with sodium laurate at any concentrations. The same results was obtained in a similar experiment when using lipid material from brain instead of lysed platelets. Sodium dodecylbenzenesulfonate had the same effects as sodium laurate.

Experiments have also been performed with an optimal concentration of eluate and varying concentrations of proaccelerin in the clotting mixtures. Sodium laurate and sodium dodecylbenzenesulfonate increased the coagulability at all concentrations of proacce-

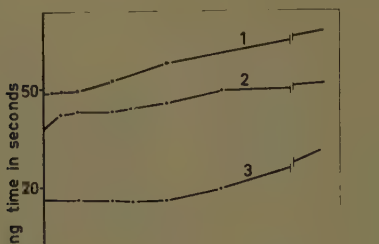



Fig. 3. Clot-promoting effect of sodium laurate in clotting mixtures with a high concentration of proaccelerin and a high concentration of lysed platelets and with different concentrations of eluate.

Clotting system: 0.1 ml eluate of different concentrations + 0.1 ml proaccelerin + 0.1 ml fibrinogen 1 % + 0.1 ml R.V.V. 1/10000 + 0.1 ml lysed platelets + 0.1 ml sodium laurate of different concentrations or physiological saline as a control + 0.1 ml $CaCl_2$ 0.04 M.

Ordinate: logarithmic scale.

1: eluate diluted 1:100 with buffered phsysiological saline.

2: eluate diluted 1:40 with buffered physiological saline.

3: eluate diluted 1:12 with buffered physiological saline.

lerin. However, when lysed platelets or lipid material from brain was present there was no shortening of the clotting times with sodium laurate or sodium dodecylbenzenesulfonate.

The conclusion which might be drawn from these experiments is that sodium laurate and sodium dodecylbenzenesulfonate either have an effect on the coagulation similar to lysed platelets and lipid material from brain or that lysed platelets and lipid material from brain inhibit the effect of sodium laurate and sodium dodecylbenzenesulfonate.

*Effect of Dodecylbenzenesulfonate in a Lipid-Poor Thrombin Generation System.*

In the next experiment, it will be shown that the effect of dodecylbenzenesulfonate on the clotting is not identical to the effect of the lipid material or lysed platelets. It was necessary to produce a lipid-poor thrombin generation mixture for this demonstration.

As probably very small quantities of lipid material are needed for the thrombin formation, it is impossible to obtain an ideal lipid free system. Our aim has been to obtain clotting systems which form no or very little thrombin without addition of extra lipid. On the other hand, there has to be a rapid thrombin formation in the system when a sufficient concentration of lipid material is added.

The clotting system used in the experiment shown in Fig. 4 was a two-stage system. The mixture in which thrombin was expected to be formed was composed as follows:

1. Eluate which had been extracted with chloroform-methanol as described above.
2. R.V.V.
3. Accelerin or proaccelerin prepared as described above.
4. Buffered physiological saline, lipid material (1.2 mg/ml), or dodecylbenzenesulfonate of various concentrations.
5. $CaCl_2$ 0.03 M.

From this incubation mixture, aliquots of 0.2 ml were taken and blown down into 0.3 ml fibrinogen 0.25 per cent at the times indicated in the figure.

The results of this experiment can be summarized as follows:

1. There was a minimal formation of thrombin in the system with accelerin with

ut extra lipid added. When lipid was added
› this system, thrombin formed sufficiently
nd rapidly. Dodecylbenzenesulfonate was,
n the other hand, not able to influence the
rombin formation.

2. If proaccelerin was used in the system
nstead of accelerin, the thrombin formation
ill was very poor. However, in this system
odecylbenzenesulfonate in a sufficient con-
entration was able to accelerate the throm-
in formation almost to the same degree as
ie lipid material.

Numerous such experiments have been
erformed with many different concentra-
ons of sodium laurate or dodecylbenzene-
ulfonate and also with lysed platelets in-
tead of the lipid material. The result illu-
trated in Fig .4 could always be equalled.

The main conclusion which may be drawn
om this experiment is that the effect of
odecylbenzenesulfonate is not identical to
ie effect of the lipid material, because dode-
ylbenzenesulfonate did not increase the
rombin formation in the accelerin system
hereas lipid material had a great accelerat-
ng effect.

*ffect of Dodecylbenzenesulfonate in a*
*hrombin Generation System with*
*Crude Accelerin".*

The most plausible explanation of the
sult of the experiment shown in Fig. 4 at
rst seemed to be that dodecylbenzenesul-
nate activates proaccelerin to accelerin.
ome observations which were made during
ie preliminary work spoke against this
eory. First, we were never able to demon-
rate any increased activity in plasma or
roaccelerin preparations with the specific

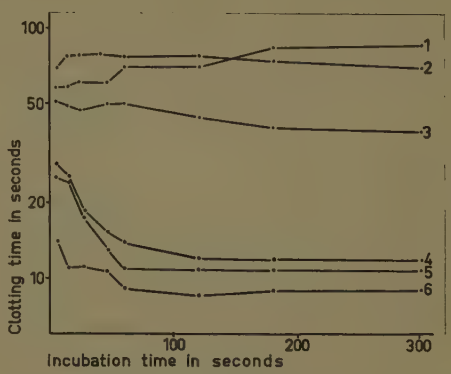



Fig. 4. Effect of dodecylbenzenesulfonate in a lipid-poor thrombin generation system with purified accelerin or proaccelerin present.

Clotting system: 0.5 ml eluate diluted 1:15 + 0.5 ml lipid material from brain, 1.2 mg per ml, dodecylbenzenesulfonate 0.14 mM, or physiological saline respectively + 0.5 ml proaccelerin or accelerin + 0.5 ml R.V.V. 1/10000 dissolved in 0.03 M $CaCl_2$.

From these incubation mixtures 0.2 ml was removed after the times indicated and blown down into 0.2 ml fibrinogen 0.25 %.

Ordinate: logarithmic scale.

1: Purified accelerin and physiological saline in the incubation mixture.
2: Purified accelerin and dodecylbenzenesulfonate in the incubation mixture.
3: Proaccelerin and physiological saline in the incubation mixture.
4: Proaccelerin and dodecylbenzenesulfonate in the incubation mixture.
5: Purified accelerin and lipid material in the incubation mixture.
6: Proaccelerin and lipid material in the incubation mixture.

proaccelerin determination method after addition of dodecylbenzenesulfonate or laurate. Secondly, we were unable to increase the thrombin formation in a lipid-poor system very much by increasing the accelerin activity a hundred times. Further, the thrombin

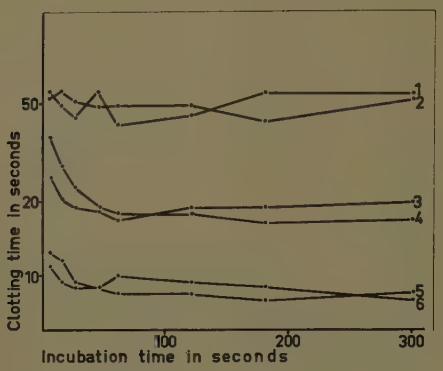



Fig. 5. Effect of dodecylbenzenesulfonate in a thrombin generation system with "crude accelerin". Clotting system: 0.5 ml eluate diluted 1:15 + 0.5 ml lipid material from brain, 1.2 mg per ml, dodecylbenzenesulfonate 0.14 mM, or physiological saline respectively + 0.5 ml proaccelerin or "crude accelerin" + 0.5 ml R.V.V. 1/10000 dissolved in 0.03 M $CaCl_2$.

From these incubation mixtures 0.2 ml was removed after the times indicated and blown down into 0.2 ml fibrinogen 0.25 %.

Ordinate: logarithmic scale.

1. "Crude accelerin" + physiological saline in the incubation mixture.
2. Proaccelerin + physiological saline in the incubation mixture.
3. "Crude accelerin" + dodecylbenzenesulfonate in the incubation mixture.
4. Proaccelerin + dodecylbenzenesulfonate in the incubation mixture.
5. "Crude accelerin" + lipid material in the incubation mixture.
6. Proaccelerin + lipid material in the incubation mixture.

generation mixture used contained R.V.V. which has been shown by Hjort (1957) to active proaccelerin into accelerin. The following experiment also definitely speaks against the theory.

A thrombin concentration which gave maximal activation of the proaccelerin preparation used was tested. It was found tha 1 N.I.H. thrombin unit per ml was suf ficient to activate the dilution of the proacce lerin-preparation used. No further increas in activity could be obtained by the additio of more thrombin. An experiment was nov performed exactly as described in Fig. 4 bu with this "crude accelerin" instead of th purified accelerin preparation. Fig. 5 show the result. The accelerating effect of dode cylbenzenesulfonate was, as will be seer quite as good with crude accelerin as wit proaccelerin.

The conclusion is that there is somethin present in the proaccelerin and "crud accelerin" preparations which is necessar for the accelerating effect of dodecylbenzene sulfonate. This component is removed by th purification of accelerin.

*Experiment with a Thrombin Generation Mixture without R.V.V. Present.*

Fig. 6 shows an experiment with a two stage system of exactly the same compositio as that shown in Fig. 4 but without R.V.V present. The experiment shows that. R.V.V is not necessary for the accelerating effect dodecylbenzenesulfonate on the thrombi formation. Similar experiments have bee performed with sodium laurate with the sam result.

## DISCUSSION

It has been known for many years that the cephalin fraction prepared from brain ar other organs there are clot promoting su stances very similar to those present platelets. During recent years (Wolf, 195 O'Brien, 1956) it has also been shown th

fied and synthetic ethanolaminglycero-
spholipid and seringlycerophospholipid
active as clot-promoting agents (Poole &
insson, 1956, Biggs & Bidwell, 1957).
great attention which has been paid to
subject is probably due to the fact that
is one of the few clotting factors which
elatively well chemically characterized.
he phospholipids which have been shown
ave activity in the blood coagulation are
phospholipids. Our working hypothesis
he beginning of this work was that the
y acids might substitute the glycerophos-
lipid, which in turn would mean that the
spholipid effect is rather unspecific. The
eriments show that the effect of the fatty
ls is not identical to the effect of the lipid
erial from brain or lysed platelets.
Contrary to the lipid material, the fatty
ls are inactive in a system where purified
elerin instead of a "crude accelerin" pre-
ation is used. The reasonable explanation
hat in the "crude accelerin" there is a
stance present which is necessary for the
elerating effect of the fatty acids.
Of the known clotting factors which are of
ortance for the thrombin formation in a
em containing R.V.V., the proaccelerin
crude accelerin preparations contain pro-
elerin-accelerin, small amounts of lipids,
perhaps inhibitors. Therefore it seems
cal to discuss the following possibilities
explain the clot-promoting activity of
um laurate and dodecylbenzenesulfonate.
Proaccelerin is activated by the anions.
s is not probable for the following
ens. First no increased proaccelerin ac-
y could be registered by the proaccelerin
rmination method after addition of so-
n laurate or dodecylbenzenesulfonate to the proaccelerin preparation. Second, the clot accelerating effect of the anions was quite as good after as before the activation of pro-

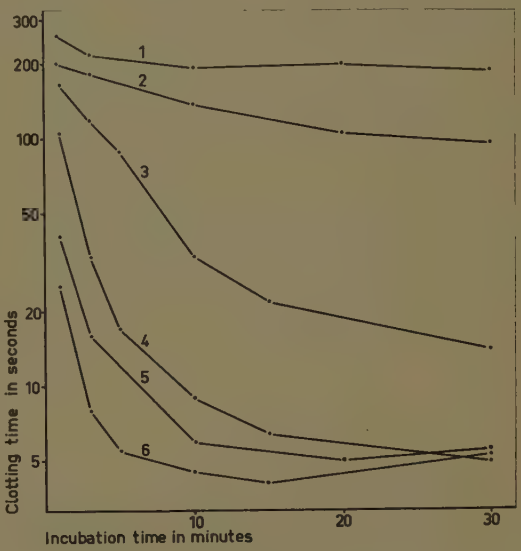



Fig. 6. Effect of dodecylbenzenesulfonate in a thrombin generation system without R.V.V.

Clotting system: 0.5 eluate undiluted + 0.5 ml proaccelerin or accelerin + 0.5 ml lipid material, 1.2 mg per ml, dodecylbenzenesulfonate 0.28 mM or buffered physiological saline respectively + 0.5 ml $CaCl_2$ 0.03 M.

After the times indicated, 0.2 ml of these incubation mixtures was removed and blown down into 0.2 ml fibrinogen 0.25 %.

Ordinate: logarithmic scale.

1: Accelerin + physiological saline in the incubation mixture.
2: Accelerin + dodecylbenzenesulfonate in the incubation mixture.
3: Proaccelerin + physiological saline in the incubation mixture.
4: Proaccelerin + dodecylbenzenesulfonate in the incubation mixture.
5: Accelerin + lipid material in the incubation mixture.
6: Proaccelerin + lipid material in the incubation mixture.

accelerin with thrombin. Third, the clotting times in the system with purified accelerin were never shortened appreciably ewen when the accelerin activity was increased a hundred times.

2. The small amounts of lipid material present in the preparations is activated by the anions, for instance by splitting of lipoprotein complexes.

3. The fatty acid and dodecylbenzenesulfonate combine with a co-factor present in the proaccelerin preparation to give an activity similar to that of the lipid material.

4. The anions neutralize a lipid factor inhibitor.

## SUMMARY

In a one-stage clotting mixture with human citrated, platelet-poor plasma and Russell's viper venom, the sodium salts of lauric acid, capric acid, caprylic acid, oleic acid, linoleic acid, cholic acid, gangliosides, and the synthetic detergents sodium dodecylbenzenesulfonate and sodium dinonylsulfosuccinate are able to promote the clotting.

Sodium laurate and sodium dodecylbenzenesulfonate are not able to influence the thrombin formation in lipid-poor thrombin generation systems when purified accelerin prepared by anion exchange resin chromatography is used. On the other hand if proaccelerin or "crude accelerin" instead of purified accelerin is used in these systems, sodium laurate and sodium dodecylbenzenesulfonate, accelerate the thrombin formation considerably. Lipid material from brain and lysed platelets have a high and similar activity in both systems.

The conclusion which has been drawn is that the effect of the fatty acids and synth detergents on the thrombin generation is identical to the effect of lysed heated pla lets or lipid material from brain. Howe together with a substance present in the p accelerin and "crude accelerin" preparati used, the fatty acids and synthetic deterge have an activity similar to lipid material lysed heated platelets.

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# PURIFICATION OF HUMAN AND BOVINE ACCELERIN (SERUM ACCELERATOR GLOBULIN) ON AN ANION EXCHANGE RESIN COLUMN

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The original observation of Ware, Murphy & Seegers (1947) and Ware & Seegers (1948) that the proaccelerin (factor V) activity of blood increases markedly during coagulation has since been confirmed by many authors (Aas, 1952, Hjort, 1957). It has been commonly accepted that the activation of proaccelerin is caused by thrombin. The active principle has been called accelerin, or serum accelerator globulin.

Lanchantin & Ware (1955) have shown that when human proaccelerin is activated by thrombin, peptide material is split off. Cox, Lanchantin & Ware (1955) have also been able to purify the active principle — accelerin — on a column of the anion exchange resin Amberlite IRA-400. They found that the activity of the purified human accelerin decreased rapidly during dialysis. The assumption was made that the active principle might be a low molecular substance. Bovine accelerin could not be purified on the Amberlite column.

In a recent publication Surgenor, Wilson & Henry (1960) have described a method for purification of proaccelerin using the Cohn fractionation technique. The authors where not able to activate their preparation with thrombin. On the contrary, thromb inactivated proaccelerin rapidly. On the ba of this observation and some kinetic studi they claim that there is no activation of p accelerin during coagulation. Thus, th results are in contrast to earlier observatio

In our laboratory we were unable to produce the method of Cox, Lanchantin Ware (1955) for purification of accele probably because of qualitative differences the Amberlite resin. A study was theref performed to investigate the possibility using other anionic exchangers to pu accelerin. It was found possible to us 20 x 50 mm column of Dowex-2. The ess tial point is that the column is short. W longer columns it was found impossible elute the accelerin activity. The method p mits purification of both human and bov accelerin. The purified principle is not ul filtrable and the activity does not dimi on dialysis.

## MATERIALS AND METHODS

*Buffered physiological saline:*

A mixture of 4 volumes of physiological sa + 1 volume of the isotonic veronal buffer of Ow (1947) pH = 7.3.

*nductivity measurements:* Performed with a ps conductivity measuring bridge GM 42 49/01 the measuring cell GM 4221 at room tempe- e. 0.2 ml of the fractions to be measured was ed with 15 ml of redistilled water and the tance was determined in these solutions.

*alysis sacks* from Viscing Corporation, Chi- , U.S.A.

*owex-2 column:* Dowex-2 x 10, 200—400 mesh v Corning Co. Michigan, U.S.A.) in the ride form was suspended in a mixture of 4 of physiological saline and 1 part of a phos- e buffer 0.05 M, pH = 7.3 and poured into a column 20 mm in diameter and allowed to nent until it reached a height of 50 mm. It was run through several times with the mixture ysiological saline and phosphate buffer 0.05 M, 7.3.

*sphate buffers:*

= 7.3 (2 ml of 0.05 M $KH_2PO_4$ + 8 ml of 0.05 M $Na_2HPO_4$)

= 6.2 (8 ml of 0.15 M $KH_2PO_4$ + 2 ml of 0.15 M $Na_2HPO_4$)

*roaccelerin (factor V):* Prepared as described Hjort (1957). 200 ml of human or bovine oxa- $BaSO_4$ — adsorbed plasma was mixed with ml of distilled water and 400 ml of a saturated, ral $(NH_4)_2\ SO_4$-solution at + 4° C, left at ° C for 30 minutes, and centrifuged. Another ml portion of the $(NH_4)_2\ SO_4$-solution was d to the supernatant. After standing for 30 tes at 4° C, the precipitate was collected by ifugation, dissolved in approximately 200 ml ysiological saline and dialysed against 10,000 f neutral distilled water for 48 hours at + 4° th 4 changes of the dialysis fluid. The slight scence which appeared during the dialysis was ved by centrifugation. To the supernatant, acid 1 per cent was added to pH = 5.5 and the solution was left at + 4° C for 30 minutes. precipitate was collected by centrifugation, lved in about 30 ml of physiological saline, and alized. It was kept in small test tubes at — 20° he preparation gave no opalescence on addition $aCl_2$. Thus it was free from $(NH_4)_2\ SO_4$.

*Proaccelerin (factor V) determination method:* The one-stage method of Aas (1952) was used. As no proaccelerin-deficient plasma was available, aged plasma prepared in the following way was used: 200 ml of human oxalated plasma was kept in a sterile, stoppered glass bottle at + 37° C for 3 days. It was then dialysed for 48 hours against 5,000 ml of a solution containing 1 volume of the veronal buffer of Owren (1947) pH = 7.3, 1 volume of 3 per cent trisodiumcitrate, and 4.4 volumes of physiological saline. The dialysis fluid was changed 5 times.

*Protein determination method:* The protein concentration in the proaccelerin preparations was calculated from the nitrogen content determined by the Kjeldahl procedure. Multiplication factor = 6.25.

The method of Lowry, Rosebrough, Lewis Farr & Randall (1951) with the Folin-Ciocalteu reagent was used for the determination of protein in the fractions. Dilutions of the proaccelerin preparations served as standards.

*Sephadex 25:* From Pharmacia, Uppsala, Sweden. Columns were made by suspending the Sephadex in distilled water and letting it sediment into a 20 x 400 mm glass column. The columns of Sephadex used were 20 x 150 mm.

*Thrombin:* Chromatographically purified human thrombin, prepared as previously described (Korsan-Bengtsen & Ygge, 1961), was used for the activation of human proaccelerin, and bovine thrombin prepared in a similar way was used for the activation of bovine proaccelerin.

*Ultrafiltration:* "Metall-Druckfiltrationsgerät MD 35-10" from Membranfilter Gesellschaft, Sartorius-Werke A.G. Göttingen, Germany with filter Lsg. 60.

## EXPERIMENTS AND RESULTS

### *Activation of proaccelerin with thrombin.*

In a test tube, 1 ml of the human or bovine proaccelerin preparations was diluted with 3 ml physiological saline, and to these mixtures, 1 ml of thrombin with an activity corresponding to 6 N.I.H. units per ml was added. Human thrombin was used for the

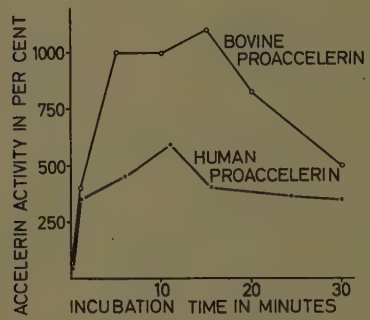



Fig. 1. Activation of proaccelerin with thrombin.

human proaccelerin, and bovine thrombin for the bovine proaccelerin. The "proaccelerin" activity in the mixtures was determined with the proaccelerin determination method described above and correlated to the activity in human undiluted plasma.

The correlation graph in the proaccelerin determination method was made from dilutions (10 per cent to 0.5 per cent) of normal human citrated plasma. To be able to read this graph, dilutions of the proaccelerin or proaccelerin-thrombin mixtures had to be made. The dilutions were always made so that they gave clotting times which could be read on the part of the graph corresponding to 10 per cent — 5 per cent of normal human plasma. To obtain this the proaccelerin-thrombin mixtures had to be diluted 100 — 200 times. The activity of normal human citrated undiluted plasma was taken as 100 per cent activity.

Fig. 1 shows the activation of human and bovine proaccelerin with thrombin in relation to incubation time.

*Separation on the Dowex-2 Column*

To 1 ml of the proaccelerin diluted with 3 ml of physiological saline, 1 ml of thrombin 6 N.I.H./ml was added as described abo After 10 minutes of incubation at 37° C whole volume was placed on the column allowed to drain in. A 20 ml portion mixture of 4 volumes of physiological sal and 1 volume of a phosphate buffer 0.05 pH = 7.3 was run through. Then the tion was performed with a solution c taining 4 volumes of NaCl 1 M and 1 volu of a phosphate buffer 0.15 M pH = 6.2.

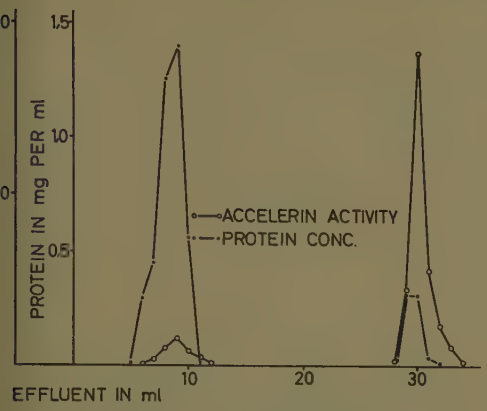
The eluate was collected in portions 1 ml and analyzed for "proaccelerin" acti and protein content. Before the determi tion of "proaccelerin" activity, the porti with high ionic strength had to be dilu with distilled water to physiological io strength and were then further diluted w buffered physiological saline.

The result of an experiment with hum accelerin is shown in Fig. 2 and with bov accelerin in Fig. 3.

As will be seen, there is no direct corr tion between accelerin activity and pro concentration in the second peak. This been a constant observation in all our exp ments and it indicates that the "accele peak" contains inactive proteins. If proa lerin which had not been activated by thr bin was run in the same way, an activity exceeding 10 per cent of undiluted, hum citrated plasma appeared together with two protein peaks.

*Removal of the Buffer and the NaCl*

The fraction from the Dowex-2 colu which contained the highest activity placed on a 20 x 150 mm column of phadex-25 which had been run through distilled water. The fraction was allowe drain in and the elution was performed




2. Separation of human "accelerin" and inactive proteins on the Dowex-2 column.

illed water. The accelerin activity apred as a single peak. For most purposes, accelerin is desired in a solution with siological ionic strength. This can be ained by running through the Sephadex umn with physiological saline and by ing with physiological saline. No further aration of accelerin and inactive proteins obtained with Sephadex-25.

### *ne Properties of the Accelerin paration*

For the following studies, a water solution accelerin, obtained from the Sephadex umn, was used.

*Dialysis:* About 5 ml of accelerin was ed in a dialysis sack and dialysed against 00 ml of distilled water at + 4° C for hours. As a control, 5 ml of the same elerin preparation was kept in a test tube he same temperature. After the dialysis, difference in accelerin activity was found veen the two samples. Thus the accelerin vity did not pass the dialysis membrane er the conditions used.

*Freeze-drying and freezing:* By freeze-drying and freezing at — 20° C, 50 per cent of the accelerin activity was lost.

*Storage at + 4° C:* By storage in a refrigerator at + 4° C, about 40—50 per cent of the accelerin activity was lost during the first 24 hours. After 8 days, about 25 per cent of the original activity still remained.

*Ultrafiltration:* About 5 ml of accelerin was placed in the ultrafiltration apparatus and exposed to a pressure of 12 Kp/cm². No accelerin activity was found in the ultrafiltrate. The dry material on the filter was redissolved in distilled water and was found to contain practically all of the original accelerin activity.

## DISCUSSION

Until now, it has been commonly accepted that proaccelerin (fact. V) is transformed into a more active principle which has been called accelerin or serum Ac-globulin during coagulation.

However, in recent work Surgenor *et al.* (1960) have, as mentioned above, prepared a proaccelerin preparation which is not activated by thrombin. A possible explanation

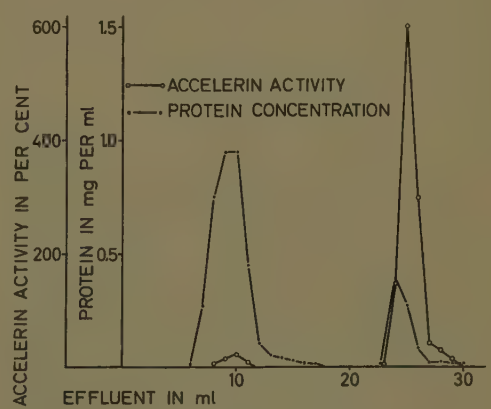



Fig. 3. Separation of Bovine "accelerin" and inactive proteins on the Dowex-2 column.

of this result might be that an activation had taken place during the preparation procedure. It has been shown by Hjort (1957) that a "spontaneous" activation of proaccelerin occurs during storage. The same observation has been made by us. We have also observed that in some of the proaccelerin preparations, only a minimal activation took place after the addition of thrombin in spite of a high original "proaccelerin" activity in the preparations.

In the present work, it has been shown that the "proaccelerin" activity in a proaccelerin preparation, tested with the proaccelerin determination method of Aas (1952), increases 10—30 times after addition of a suitable concentration of thrombin. The activated proaccelerin preparation could be separated on an anion exchange resin and gave a highly active and protein-pure preparation. If, on the other hand, unactivated proaccelerin was run on the anion exchange resin column, only minimal activity appeared, mainly together with the bulk of the protein. These observations can hardly be explained if there is no activation of the proaccelerin preparation by thrombin. Thus our results are in accordance with those of Cox *et al.* (1955).

## SUMMARY

The optimal conditions for the separation of accelerin on anion exchange resins have been investigated. A short column of Dowex-2 was found to be suitable. The essential point is to use a short column, as otherwise it was not possible to elute the active principle. The method admits preparation of both human and bovine accelerin in contrast to what was found by Cox *et al.* (1955). The removal of the buffer is easily performed a Sephadex-column.

The accelerin prepared in this way is p bably not a low molecular substance, beca it passes through the Sephadex column gether with the protein, it is not ultraf rable, and does not pass the dialysis me brane.

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# A ONE STAGE METHOD FOR THE ASSAY OF PLATELET FACTOR 3

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The term platelet factor 3 was first app-
d by van Creveld & Paulssen (1951) to
note an activity in the sediment of plate-
extracts. This factor possessed anti-
parin activity, but "thromboplastin" activ-
was also present. Jürgens (1952) and
utsch (1954) separated these two activ-
s, and retained platelet factor 3 as the
ne for the thromboplastic factor; the anti-
parin factor was numbered 4. Factor 3
ms to reside in the granulomere of blood
telets (Fonio 1951) and is of lipoprotein-
ure (Shinowara 1957). It is a component
intrinsic prothrombinase or blood throm-
plastin (Biggs & Douglas 1953).

Various methods have been proposed for
assay of this factor. They are mainly
p-stage methods based on the thrombo-
stin generation test of Biggs & Douglas
953). Generally, these methods are rather
e-consuming and at best semi-quantita-
. Conflicting reports on the behavior of
elet factor 3 in the heterogenous group
"thrombocytopathies" probably reflect the
iability and lack of accuracy of the assay
hods employed.

The purpose of this investigation was to
elop a one-stage assay method based on the same principle as one-stage assays of plasma clotting factors. In such tests, sensitivity and specificity are usually obtained by using diluted test materials and a substrate which contains all factors in high and constant amounts, except the one to be tested. In the present assay system platelet-free human plasma is used as the substrate, and diluted plasma with a known number of platelets as the test material. The system is simple and has the same accuracy as the corresponding assays of plasma clotting factors.

## MATERIALS

*Calcium chloride.* A 35 mM solution in distilled water was optimal in the system.

*Celite powder,* "Speedex", which is amorphous diatomaceous $SiO_2$ from Dicalite Divisions' Great Lakes Carbon Corp., New York, was used.

*Cephalin suspension.* The method is described by Hjort, Rapaport & Owren (1955).

*Citrated blood.* Venous blood was drawn with siliconized needles into siliconized tubes. The first 5 ml of blood were discarded, and nine parts of blood were then allowed to flow directly into the tube containing one part of 3.13 per cent sodium citrate dihydrate.

*Dilution fluids I and II* were prepared as described by Hjort *et al.* (1955). All test plasmas were first diluted in dilution fluid I to a concen-

tration ten times the desired one. The final 1:10 dilution was done in dilution fluid II. This technique secures a constant concentration of citrate and ionic strength in all plasma dilutions.

*Adsorbed platelet-free bovine plasma.* Nine parts of blood were mixed with one part of 2.5 per cent potassium oxalate monohydrate and centrifuged at 2,500 r.p.m. (1,300 G) for 30 minutes at 4° C. The plasma was pipetted off and adsorbed by filtering, first through a 20 per cent asbestos filter pad (filter diameter 15 cm, oxygen pressure about 1 kg per square cm, filter changed every 500 ml) and then through a 50 per cent asbestos filter (oxygen pressure 1.5—2 kg per square cm, filter changed every 300 ml). The filtered plasma was dialyzed overnight at 4° C. against frequent changes of saline. To remove remnants of platelet material, the plasma was then centrifuged in 12 ml lusteroid tubes at 40,000 r.p.m. (144,700 G) for 60 minutes at 40° C. The plasma in the middle part of the tubes was collected as described below for platelet-poor human plasma. It was stored in acid-cleaned stoppered tubes at —20° C. Before use, it was diluted to 25 per cent in dilution fluid II.

*Platelet-poor human plasma.* This is the substrate plasma in the assay system. A sensitive and specific assay system requires a nearly complete removal of platelets. Essential steps in achieving this are: low temperature (about 4° C.) throughout the procedure, high speed centrifugation, and above all a correct pipetting technique from the tubes after high-speed centrifugation.

For each batch of substrate plasma, two fasting blood donors were used. Citrated blood was obtained by the method described, 450 ml blood from each donor divided in two 250 ml bottles. The blood was centrifuged at 2,500 r.p.m. (1,700 G) for 30 minutes at 4° C. The plasma was pipetted off and centrifuged in 12 ml lusteroid tubes at 40,000 r.p.m. (144,700 G) for 60 minutes at 4° C. A small sediment of platelets was observed, and a thin fatty film was formed at the plasma surface. Obviously, the bottom layer must be avoided when collecting the plasma, but it is extremely important also to avoid the top layer, not because of the chylomicrons present, but because this layer contains a considerable number of platelets. Usually 1,000—5,000 platelets per $mm^3$ were counted in the t layer, whereas the middle layer had less than 50

A needle was carefully introduced through t centre of the top layer, and plasma from t middle part of the tube was aspirated into a s conized syringe, leaving the top- and bottom lay in the tube. Only ⅔ to ¾ of the plasma was us The needle was carefully wiped off with a fil paper before aspirating a new tube. Plasma fr all tubes was collected in 4 separate portio A small sample from each portion was frozen a thawed twice, activated (see below) and tested the following system: 0.2 ml activated plasma 0.2 ml filtered bovine plasma diluted 1:4 in dilut fluid II + 0.2 ml dilution fluid II. The mixture w incubated at 37° C. for 3 minutes, recalcified w 0.2 ml calcium chloride, 35 mM, and the clott time recorded. The clotting time was usually b tween 240 and 300 seconds. If it wass less than 2 seconds, the portion in question was re-centrifug at 40,000 r.p.m. for 30 minutes, and the middle lay collected as described above. The plasma portio were finally frozen and thawed, pooled, distribut in aliquots of 1—2 ml in acid-cleaned, stoppe tubes and stored at —20° C.

*Platelet-rich standard plasma.* Ten ml of citra blood was obtained from each of ten normal p sons. The samples were centrifuged at 600 r.p (70 G) for 30 minutes at 4° C. The plasmas w pipetted off and mixed. The platelets were coun in the pooled plasma. It was then distributed in ml portions in acid cleaned tubes, and frozen a thawed twice before testing. This source of platel was used for the construction of the correlat graph and for the control of the stability of system. The standard plasma was stored at —2

*Platelet suspensions* were prepared from citra blood or from EDTA-blood.

a) Citrated blood was obtained from 6 nor persons, 10 ml from each. It was centrifuged at r.p.m. (70 G) for 30 minutes at 4° C. The plas were pipetted off, mixed, and centrifuged at 2, r.p.m. (1,300 G) for 30 minutes at 4° C. The pl let-poor plasma was discarded, and the plat sediment resuspended with a Cronkite's resus sion pipette, and washed 3 times with 5 ml aliq of ice-cold dilution fluid II. The platelets w

lly resuspended in 5 ml of the same fluid until omogeneous suspension was obtained. It con-ed about 800,000 platelets per mm³. The suspen-was distributed in aliquots of 0.2 ml and frozen thawed twice before testing.

) Nine ml of blood from each of 6 normal per-s were mixed with 1 ml of an isotonic solution per cent ethylene-diaminetetraacetate in saline solution was adjusted to pH 7.3 with N/2 OH). Platelet suspension was prepared as de-bed above, except that the first two washings re performed with one part of the anticoagulant five parts of saline. The suspension contained ut 2,000,000 platelets per mm³.

## METHODS

*Activation* of platelet-poor substrate plasma was ried out immediately after thawing a sample of 2 ml, with 30 mg of celite per ml of plasma. is amount gave maximum activation. The celite-sma suspension was tilted at room temperature 10 minutes. The celite powder adsorbs the bulk he activation product (Waaler 1959), and it was removed. The activated sample was kept in water until the tests were performed.

*Disintegration of platelets* was performed by ezing and thawing. Aliquots of 0.2 ml were tted into 3 ml test-tubes and frozen and thawed —20° C. and 37° C., respectively, and then roughly mixed with dilution fluids I and II in proportions given.

*Platelet counting.* The method of Brecher & nkite (1950) was used. When preparing the relation graphs, two counting pipettes and two nting chambers were used for each plasma, nting between one and two thousand platelets gether. According to Berkson, Magath & Hurn 40), the coefficient of variation is then 5.1—5.5 cent, a result which agrees with earlier studies his laboratory (Hellem 1960).

*The test* was done by mixing 0.2 ml of activ-platelet-poor plasma + 0.2 ml of adsorbed, h-speed centrifuged bovine plasma diluted 1:4 dilution fluid II + 0.2 ml of test-material in ropriate dilution. The mixture was incubated at 37° C. for 3 minutes, 0.2 ml of calcium chloride, 35 mM, was added, and the clotting time recorded. The end point, the formation of a solid clot, was sharp and easy to read. All tests were done in duplicate.

Table I. *Determination of the optimal amount of celite for activation of the substrate plasma.*

| Amount of celite in mg per ml of substrate plasma | Clotting time in sec. | |
|---|---|---|
| | 1/100 | 1/1,500 |
| 5 | 59.0 | 145 |
| 10 | 54.6 | 133 |
| 15 | 53.4 | 132 |
| 20 | 54.0 | 130 |
| 25 | 52.4 | 127 |
| 30 | 52.8 | 125 |
| 35 | 52.8 | 125 |
| 40 | 52.8 | 125 |
| 50 | 53.6 | 126 |
| 60 | 53.8 | 127 |
| 70 | 55.0 | 133 |
| 80 | 59.0 | 140 |

The activation was performed as described under methods. The clotting system was: 0.2 ml of activated platelet-poor plasma + 0.2 ml adsorbed high-speed centrifuged bovine plasma diluted ¼ in dilution fluid II + 0.2 ml diluted platelet-rich test-plasma frozen and thawed twice + 0.2 ml calcium chloride, 35 mM. Two dilutions of the test plasma were used, 1/10 and 1/1,500.

## RESULTS

*The optimum amount of celite for the activation of the substrate plasma.* This was found, by the experiments presented in Table I, to lie between 20 and 60 mg per ml of plasma. Although in the lower half of the range for maximum activation, 30 mg per ml was chosen as it was more convenient to perform the test with a relatively small amount of celite powder.

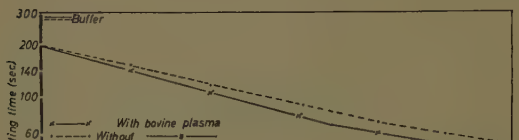



Fig. 1. *The effect of bovine plasma in the system.* The test system was: 0.2 ml activated platelet-poor plasma + 0.2 ml filtered high-speed centrifuged bovine plasma diluted ¼ in dilution fluid II (or dilution fluid II only) + 0.2 ml platelet-rich test plasma frozen and thawed twice + 0.2 ml calcium chloride, 35 mM.

*The value of adsorbed high-speed centrifuged bovine plasma in the system.* This is shown in Fig. 1, which presents the correlation graph (see below) with and without bovine plasma. At the lowest concentration of platelets in the test plasma, bovine plasma did not accelerate coagulation. This means that the bovine plasma used did not contain platelet factor 3. The accelerated clotting produced by bovine plasma at higher concentrations of platelet factor 3 was due to the increased concentrations of nonadsorbable plasma clotting factors such as factor V and VIII, provided by the bovine plasma. These factors were present in suboptimal amounts in human plasma for this technique, and addition of adsorbed bovine plasma increases reliability and stability.

*The effect of freezing and thawing on the release of platelet factor 3.* When platelet-rich plasma was tested immediately after centrifuging before freezing and thawing, the platelets were nearly inactive in the system. Freezing and thawing produced a 100—200 fold increase of activity. Table II shows that one freezing and thawing was nearly sufficient to release the factor. After the seco freezing and thawing, no further increase activity could be obtained.

*The stability of the reagents at 4° C.* T activated substrate plasma and the bov plasma were stable for at least 5 hours ice-water as shown in Table III. Usually, plasma was used within 1—2 hours after activation procedure.

*Stability of the substrate plasma and st dard plasma on storage at —20° C.* C batch of substrate plasma usually lasted 1 months. Table IV shows that this plasm judged by the buffer time of the syst (dilution fluid II instead of test materi was stable at —20° C. at least for this ti The clotting times of various dilutions of standard plasma show that platelet factor when stored under these conditions was a

Table II. *The effect of repeated freez and thawing on the release of platelet factor 3.*

| Number of freezing-thawing procedures | Clotting time in sec. |
|---|---|
| 0 | 193.2 |
| 1 | 71.7 |
| 2 | 69.8 |
| 3 | 70.5 |
| 4 | 69.9 |
| 5 | 69.8 |
| 6 | 68.7 |
| 7 | 69.2 |

A platelet-rich test plasma was distributed in 0.2 aliquots in acid-cleaned test tubes. One sample diluted 1/50 and tested in the system. The ot samples were frozen at —20° C. and thawed 27° C. for the given number of times, diluted and tested. The system was: 0.2 ml activated pl let-free plasma + 0.2 ml dilution fluid II + 0.2 diluted test plasma + 0.2 ml calcium chlor 35 mM.

ble III. *Stability on storage at 4° C. of eagents in the assay system for platelet factor 3.*

| Hours of storage | Clotting time in sec. | |
|---|---|---|
| | Test plasma | Dilution fluid II |
| 0 | 57.1 | 252 |
| 1 | 56.2 | 250 |
| 3 | 56.0 | 249 |
| 4 | 57.2 | 248 |
| 5 | 56.7 | 252 |

vated substrate plasma and bovine plasma were ared as described and stored in stoppered tubes e-water. The clotting system was as described able I. In one set of tests platelets-rich plasma en and thawed twice and diluted 1/100 was used st material. In another set, the test plasma was tituted by dilution fluid II. A new sample of plasma was thawed each hour and tested against the stored reagents.

le. Thus, the same standard plasma could used for subsequent batches of substrate ma. Reproducibility of results could thus chieved.

*reparation of the correlation graph and ction of a suitable dilution of the test ma.* The correlation graph was obtained esting various dilutions of the standard ma, and plotting number of disintegrated elets against clotting times in seconds, as vn in Fig. 2. The curve forms nearly a ght line on double logarithmic paper be- n 40—50 seconds and 180—200 seconds, esponding to a range from about 10,000 00 disintegrated platelets per mm³ of the samples. Usually there is a slight bend concentration of 3,000—5,000 platelets mm³. The buffer time for this particular trate plasma was 270 seconds. Conse- tly, even activities below 100 disinte- grated platelets per mm³ could be measured. Below 40—50 seconds the curve flattened out. Thus the sensitivity is reduced in this range. A sample for testing should contain 100—4,000 platelets per mm³. The percentage activity of the platelets tested is calculated from the number of the platelets in the standard plasma and the test plasma giving the same clotting time.

*The activity of platelet suspensions in the system.* Fig. 3 shows the correlation graphs for two different platelet suspensions and for platelet-rich plasma. The platelet suspension prepared from EDTA-plasma showed lower clotting activities than the suspension from citrated plasma. Platelets in plasma showed activities in between. Resuspension of the platelet sediment was more difficult with citrate than with EDTA, and consequently platelets could have disintegrated in the citrate suspension and escaped counting. A second possible cause of the difference in activity recorded may be related to differences in the particle size of the platelet factor released.

Table IV. *Stability of substrate plasma and standard plasma on storage at —20° C.*

| Dilution of standard plasma | Storage time (days) | | |
|---|---|---|---|
| | 1 | 32 | 65 |
| | Clotting time in sec. | | |
| 1/10 | 33.6 | 32.8 | 32.8 |
| 1/20 | 35.9 | 35.8 | 36.2 |
| 1/50 | 42.9 | 42.5 | 43.4 |
| 1/150 | 56.6 | 56.6 | 57.0 |
| 1/500 | 79.4 | 80.8 | 79.4 |
| 1/1, 500 | 113 | 116 | 116 |
| 1/5, 000 | 167 | 172 | 170 |
| Dilution fluid II | 262 | 258 | 259 |

The clotting system was as in Table I.

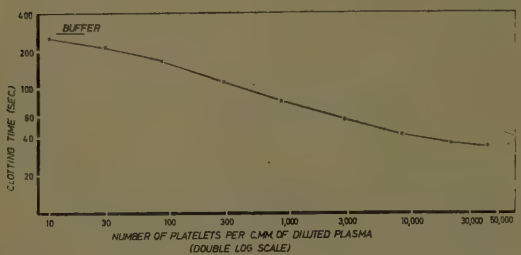



Fig. 2. *Standard dilution curve.* Number of disintegrated platelets in the samples of diluted standard plasma is plotted against clotting times on double logarithmic scale. Clotting system as in Table I.

*Sensitivity to cephalin.* Cephalin can substitute for platelets in the formation of intrinsic prothrombinase as shown by Bell & Alton (1954). Fig. 4 shows that the present system was sensitive to cephalin, and dilutions of cephalin gave a correlation graph similar to that of plasma with disintegrated platelets.

*Insensitivity to plasma clotting factors in the test material.* It is unlikely that plasma clotting factors in the test plasma could influence the system, as the plasmas were

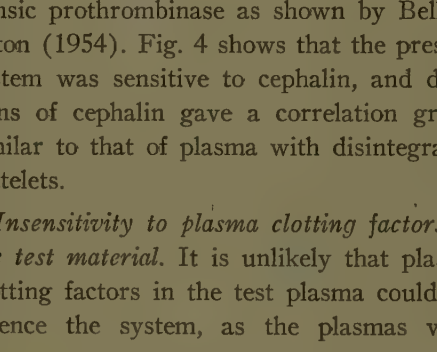



Fig. 3. *The activity of platelet suspensions in the assay system for platelet factor 3.* Correlation graphs were prepared with 3 different test materials. a) Platelet suspension prepared from EDTA-plasma. b) Platelet-rich citrated plasma frozen and thawed twice. c) Platelet suspension prepared from citrated plasma.

usually diluted 1:50 or more before testin
Lack of plasma clotting factors in the te
plasma proved to be of no importance. Pla
let factor 3 was assayed in plasma fro
patients with hemophilia A and B, from
patient with congenital lack of factor V, a
in patients treated with dicumarol, with no
man results.

*Normal variation.* Correlation graphs we
prepared as described above with platele
rich plasma from each of 25 normal perso

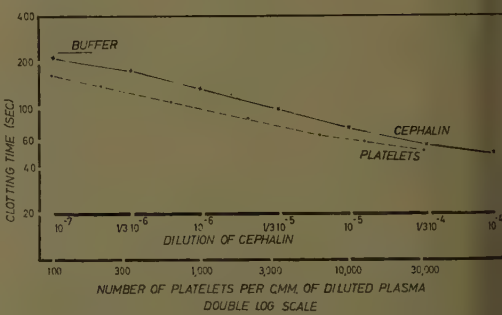
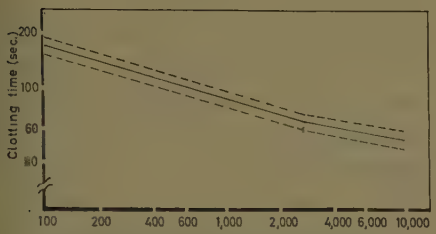



Fig. 4. *The activity of a cephalin suspension compared with platelet-rich standard plasma.* S tem: 0.2 ml activated substrate plasma + 0.2 dilution fluid II + 0.2 ml cephalin suspension, platelet-rich plasma frozen and thawed twice 0.2 calcium chloride, 35 mM.

as test plasma. The mean and range clotting times at various platelet concentr tions are shown in Table V and Fig. 5. T coefficient of variation was 5—6 per cent. can be calculated from the curve that for constant number of platelets, the activity platelet factor 3 varies between 60 and 1 per cent, *i. e.* about the same variability for some of the plasma clotting factors. T is shown in Table VI.

Table VII shows the variability of resu




. 5. *Correlation graphs from 25 normal persons.* an and range. Clotting system as in Table I.

different samples from one person. Eight nples of platelet-rich plasma were tested er a period of 6 weeks. One sample showed activity of 108 per cent, the others be- een 71 and 84 per cent.

## DISCUSSION

The activity of platelet factor 3 is related the cephalin content of the platelets, as own by Chargaff, Bancroft & Stanley- own (1936). However, there is disagree- nt concerning the specific cephalin com- und responsible: phosphatidyl ethanol- ine (O'Brien 1956, Poole & Robinson 56, Barkan, Newland & Wild 1956) or osphatidyl serine (Troup & Reed 1958, oup, Reed, Marinette & Swisher 1960, arcus & Spaet 1958). Probably, maximum ivity depends upon a mixture of these two d possibly other substances in certain pro- tions. The activity also seems to depend particle size (Boyles 1959), a fact which obably explains variations in activity of pensions prepared from identical sources. present, biochemical methods can there- e not be used for the assay of platelet fac- 3, and it must be measured on the basis its clotting activity.

Various systems have been proposed. The thromboplastin generation test of Biggs & Douglas (1953) applying suspensions of washed platelets is most widely used. The preparation of such suspensions is time-consuming, and sometimes difficult, especi-

Table V. *Normal variations in platelet factor 3.*

| Clotting time in sec. | Number of patelets | | | |
|---|---|---|---|---|
| | 10 000 | 3 000 | 1 000 | 200 |
| Range | 46–58 | 58–72 | 78–97 | 126–155 |
| Mean | 52 | 66 | 88 | 139 |
| Standard deviation (SD) | 2.6 | 3.9 | 5.4 | 7.2 |
| Coefficient of variation | 5.0 | 5.9 | 6.1 | 5.2 |
| Mean ± 2 SD | 47–57 | 58–74 | 77–99 | 125–153 |

Platelet-rich plasma from each of 25 normal persons were tested in different dilutions and correlation graphs prepared. The clotting times at 4 different levels of platelets in the diluted plasma were obtained from the individual dilution curves. The data from all persons are collected in the table.

Table VI. *The variability of platelet factor 3 activity.*

| Number of platelets (S) | Clotting time (Mean) | Range of platelet numbers with same clotting time (T) | Variability of platelet factor 3 concentration in per cent (100 S/T) |
|---|---|---|---|
| 10 000 | 52.5 | 6 100–17 500 | 57–164 |
| 5 000 | 60 | 3 200– 8 400 | 60–156 |
| 2 000 | 73 | 1 300– 3 100 | 65–154 |
| 1 000 | 88 | 680– 1 580 | 63–147 |
| 300 | 125 | 200– 410 | 73–150 |

The mean of clotting times at 5 different levels of platelets were calculated from the standard dilution curves of 25 normal persons. The range of platelet numbers with the same clotting activity is read from Fig. 5.

Table VII. *The reproducibility of results in the assay system of platelet factor 3 in the same person from day to day.*

| Test No. | Number of platelets per mm³ of diluted test plasma | Clotting time in sec. | Number of platelets in standard plasma with same clotting time | Activity in per cent |
|---|---|---|---|---|
| 1 | 2 870 | 71.8 | 2 250 | 78 |
| 2 | 2 550 | 73.3 | 2 100 | 83 |
| 3 | 3 090 | 68.3 | 2 600 | 84 |
| 4 | 2 980 | 72.3 | 2 150 | 72 |
| 5 | 2 950 | 73.1 | 2 100 | 71 |
| 6 | 3 150 | 68.4 | 2 600 | 83 |
| 7 | 2 700 | 66.7 | 2 900 | 108 |
| 8 | 2 130 | 76.0 | 1 850 | 81 |

Citrated platelet-rich plasma was obtained from the same person on 8 different days in the course of 6 weeks. The plasma was frozen and thawed twice and tested in dilution 1/150.

ally in thrombocytopenia. They contain a mixture of intact and disintegrated platelets in various proportions depending on the technique applied. Disintegrated platelets are active in the clotting system, but escape platelet counting, and consequently introduce an error in the correlation of platelet number and clotting activity.

Another two-stage test is based on the activation of purified prothrombin by platelet extract, calcium and an antihistaminic (Benadryl) (Alkjaersig, Abe & Seegers 1955). It is even more complicated, because it includes purification of prothrombin.

Methods based on prothrombin consumption have largely been abandoned because of inaccuracy.

A one-stage test based on bovine substrate plasma was recently introduced by Stapp (1959). The sensitivity of this method seems to be low, and standardization of the contact factor difficult to achieve. This is a cruci point in all methods involving the intrins blood clotting system. Experience has show that methods based on *non*-activated reagen and test plasmas are extremely sensitive small variations of contact, and in spite meticulous technique the reproducibility results is usually poor, except in the inve tor's hands. In the present test therefore, t contact factor was short-circuited by intr ducing a maximally pre-activated substra plasma. By this technique, the error caus by varying activation of the test material also eliminated.

One particular technical point must stressed in obtaining the substrate plasma platelet-poor as possible. When the plasm is collected after high-speed centrifugatio it is important not only to avoid the platele rich bottom layer, but also the fatty top laye as mentioned, because this layer contains considerably number of platelets (Huso 1961). Adsorbed bovine plasma was add to the substrate plasma in order to provi high and constant concentrations also of t non-adsorbable factors, especially V a VIII, which were found to be present in su optimal concentrations in human plasma f this method. The *specificity* of the syste seemed to be satisfactory as demonstrated the testing of plasmas deficient in differe clotting factors, and by comparing the effe of platelet materials and brain cephalin. T *sensitivity* of the system is fairly hig Amounts of platelet factor 3 correspondi to as little as 10—20 platelets per mm³ c be detected.

The main advantage of testing platelets their own plasma is that disintegration essentially avoided. The average activity

n platelet can therefore be measured. In pension of platelets separated from plas- the presence of an unknown number of nitegrated platelets, which cannot be nted, will make such calculations less able. Activity of platelet factor 3 in plate- from 25 normal persons, as calculated platelet, varied from 60—160 per cent. s variation is of the same magnitude as the antihemophilic factors. A part of this iation is technical as shown by the varia- of resuls in repeated tests from the same son. Variations must be expected as the involves platelet counting and a clotting , both of which have their own varia- ty.

One of the objects of this test was to study heterogenous group of so-called throm- ytopathia, presumably caused by partial of platelet factor 3 (Braunsteiner 1955), by an increased platelet resistence, with icient release of this factor (Johnson, nto & Caldwell 1958). The application of test to platelets in these disease groups n progress.

## SUMMARY

A simple one-stage method for the assay platelet factor 3 is described. It is based the recalcification time of activated plate- free human plasma enriched with factor nd VII through the addition of adsorbed elet-free bovine plasma. The platelets are ed in their own plasma in high dilutions. method is sensitive and specific. Errors sed by a varying contact activation dur- preparation of the substrate plasma and formance of the test are eliminated. Plate- actor 3 in relation to the number of plate- has been examined in 25 normal persons. The factor varied between 60 and 160 per cent. The reproducibility of the results in the same person from day to day was acceptable.

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# HE EFFECT OF HUMAN CHYLOMICRON SUSPENSIONS ON HE RECALCIFICATION TIME OF PLATELET-POOR PLASMA: AN EFFECT DUE TO BLOOD PLATELETS

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The effect of plasma lipids on blood coaation has attracted considerable attention ing the last decade, and there is a tenıcy to associate accelerated clotting times uced by fat with a disposition to thromis.

The problem has been studied in two prinals ways. In the first, fatty meals were en to human subjects, and pre- and postndial samples of blood or plasma were npared in various clotting tests. In the ond, the effect of fatty substances added plasma *in vitro* were studied.

n the first group of studies, the clotting e of plasma in the presence of Russel's er venom or the clotting time of whole od in silicone coated tubes have been nly used as an index. Most investigators e that the Russell's viper venom acceted clotting time is shortened after a fatty l (Fullerton & Anastasopoulus 1949, Brien 1955, Maclagan & Billimoria 1956, rskey & Nossel 1957), but no effect has been reported (Lewis 1960). An association between alimentary lipemia and hypercoagulability in the silicone clotting time of whole blood has been claimed by a number of investigators (Fullerton, Davie & Anastasopoulus 1953, O'Brien 1955, Keys, Buzina, Grande & Anderson 1957), but denied by an almost equal number (Tulloch, Overman & Wright 1953, Sohar, Rosenthal & Adlersberg 1957, Sheehy & Eichelberger 1958, Lewis 1960).

The early work in the second group of studies also applied the Russell's viper venom accelerated clotting time. Macfarlane (1941) showed that it was prolonged when particulate fat was removed by high speed centrifugation, and shortened when the fatty supernatant layer was added.

In recent years more interest has been attached to the role of chylomicrons in clotting systems not involving Russell's viper venom. Poole (1955) showed that the recalcification time of human plasma freed of particulate fat by high speed centrifugation was prolonged and could be brought down

to control level by the addition of a washed suspension of chylomicrons removed by centrifugation. This effect was attributed to components of the chylomicrons, at it was considered most unlikely that blood platelets were present in these suspensions. Essentially similar results were reported by Kingsbury & Morgan (1957). As a result of these observations, efforts have been directed towards the identification of the active coagulant substance in chylomicrons (Poole & Robinson 1956, Robinson & Poole 1956).

During attempts at preparing a very platelet-poor substrate plasma for the assay of platelet factor 3, I also found that the fatty layer after high speed centrifugation had a shorter recalcification time than the clear middle layer. By examining samples from the different layers in the phase contrast microscope, many more platelets were observed in the top layer than in the middle layer. This led to a quantitative study of the coagulant effect of the top layer in order to determine whether this effect was due to platelets, to chylomicrons, or to both. The results indicate that the whole effect could be explained by the blood platelets present.

## MATERIALS AND METHODS

*Assay of platelet factor 3.* The method has been described elsewhere (Husom 1961). It is a one stage system based on the effect of the test material on the recalcification time of a nearly platelet-free, activated human plasma. The presence of high and constant amounts of non-adsorbable clotting factors in the substrate plasma was secured by the addition of adsorbed bovine plasma. The standard for the construction of a dilution curve was a platelet-rich plasma with a known number of platelets which was frozen and thawed twice to release platelet factor 3. The curve was a straight line on double logarithmic paper between 40—50 seconds and 180—200 seconds. The corresponding concentrations o platelet factor 3 were equal to about 10,000 to 10 lyzed platelets per $mm^3$ in the dilutions used. Th lowest concentration gave a marked shortening o the recalcification time, thus allowing assay o activities also below 100 lyzed platelets per mm Intact platelets were nearly inactive in this systen

*Chylomicron and platelet suspensions.* Blood wa drawn with silicone technique from normal perso under the following 3 conditions: (1) fasting, (2 2—3 hours after an ordinary breakfast and (3 after a breakfast including 300—600 ml of 30 pe cent cream. The blood samples were mixed wit 1/10 the volume of 3.13 per cent sodium citrate di hydrate and centrifuged at 2,500 r.p.m. (1,300 G for 30 minutes at 4° C. The platelet-poor plasm was pipetted off and centrifuged in 12 ml lusteroi tubes at 40,000 r.p.m. (144,700 G) for 60 minute The fasting plasma samples showed a very thi slightly opaque surface film, whereas the post prandial samples, especially after the cream break fast, had a thick milky top layer, measuring 2— mm. along the wall of the tube. The samples wer subsampled as follows: (1) the top layer wa pipetted off in an amount of 0.5 to 0.8 ml. (2) Par of the middle layer was collected by the use of syringe and needle, carefully avoiding admixtur from the top layer. (3) The small platelet butto was resuspended in 1 ml of the bottom layer of th plasma. Platelets were counted in the samples fro the top layer and in some cases also from the middl layer. The samples were then frozen and thawe twice and tested in the assay system describe A number of non-frozen samples were also teste

*Platelet counting.* The counting chambers wer filled with undiluted samples and left for 30 minute in a moist chamber. Platelets and chylomicrons the separated in two main layers, platelets at th bottom and chylomicrons at the top. Two countin chambers were used for each sample, and abo 500 platelets were counted by the use of phase co trast microscopy. According to Berkson's formul (1940) the coefficient of variation was then 5.3 p cent, and this is in accordance with earlier studi in this laboratory (Hellem 1960).

able I. *Recalcification times of different ers of normal plasma after high speed centrifugation.*

| Sample after: | Clotting time in sec. | | | | | |
|---|---|---|---|---|---|---|
| | Top layer | | Middle layer | | Bottom layer | |
| | Intact | Frozen-thawed | Intact | Frozen-thawed | Intact | Frozen-thawed |
| ting | 104 | 62 | 250 | 242 | 60 | 36 |
| ty meal | 126 | 80 | 265 | 258 | 54 | 37 |

e samples were activated with celite, 30 mg per and tested as follows: 0.2 ml activated sample 0.2 ml filtered high speed centrifuged bovine sma diluted 1 in 4 in dilution fluid II + 0.2 ml ution fluid II. The mixture was incubated for 3 utes at 37° C., 0.2 ml calcium chloride, 35 mM was added, and the clotting time recorded.

## RESULTS

Table I shows the recalcification times of various layers after high-speed centri- gation of platelet-poor plasma. The recalci- ation time of the top layer was consider- y shorter than that of the middle layer. e bottom layer showed the shortest time. e recalcification time was shortened by ezing and thawing in all three layers, al- ugh very little in the middle layer. The le also shows that the slightly opaque top er from a fasting sample had a shorter alcification time than the heavily opaque er obtained after a fatty meal. Both top ers however, contained a clot accelerating nciple which was not present in the middle er. These results suggested that the clot moting activity could not be exerted by the amount of chylomicrons present. The effect of freezing and thawing pointed to an effect caused by cells, presumably platelets.

Therefore, the various layers were examined for platelets by phase contrast microscopy after storage of the counting chambers for 30 minutes as described. Numerous platelets were found in samples from the top layer, but very few in the middle layer. The chylomicrons were also easily observed, having risen to the top of the counting chamber.

Top layers from plasmas with various amounts of chylomicrons were diluted 1 in 10 and tested in the assay system for platelet factor 3. Table II shows clotting times and number of platelets in the various samples. For comparison, the last column gives the range for the number of lyzed platelets in plasma relatively free of chylomicrons, which gave identical clotting times in the same system. This range was obtained by analyzing a number of standard dilution curves prepared by the use of platelet-rich fasting plasma from 25 normal persons (see methods). It will be seen that the clotting activity of the top layer corresponds very well to the activity of the number of platelets present. The table also indicates that non-frozen samples have very low activity as compared with frozen and thawed samples. Sample No. 10 is of particular interest. It was taken from a patient with severe idiopathic thrombocytopenia (3,500 platelets per $mm^3$ in the blood) 3 hours after a fatty meal. The chylomicron-rich top layer contained only about 30 platelets per $mm^3$. A 1 in 10 dilution showed a clotting time of 237 seconds. The "blank" time was 248 seconds. Thus the large amount of chylomicrons exerted no clotting activity because the very low activity

Table II. *The clotting activity of top layers after high speed centrifugation of plasma.*

| Sample no. | Clotting time in sec. | Platelet number per mm³ of diluted (1/10) test material | Range of platelet numbers with same clotting activity |
|---|---|---|---|
| 1 | 141 | 190 | 115–220 |
| 2 | 127 | 310 | 165–310 |
| 3 | 139 | 140 | 125–240 |
| 4 | 108 | 470 | 270–490 |
| 5 | 131(210) | 256 | 145–280 |
| 6 | 166(220) | 82 | 56–130 |
| 7 | 167(222) | 80 | 54–130 |
| 8 | 168(228) | 86 | 54–130 |
| 9 | 182(222) | 70 | 36– 90 |
| 10 | 237 | <10 | <10 |

The assay system for platelet factor 3 was used: 0.2 platelet-free, activated human plasma + 0.2 ml filtered high speed centrifuged bovine plasma diluted 1 in 4 in dilution fluid II + 0.2 ml of test material frozen and thawed twice, and diluted 1 in 10 in dilution fluid II + 0.2 ml calcium chloride, 35 mM. Clotting times for test samples which were not frozen and thawed are given in brackets. Samples 1—4 were from fasting persons, 5—7 were drawn 3 hours after an ordinary breakfast, and samples 8 and 9 after a breakfast with an extra 600 ml of cream. Sample 10 was taken from a patient with severe thrombocytopenia 3 hours after a fatty meal.

See text for explanation of the last column.

measured corresponded to the activity of the few platelets present.

In these experiments all test samples were diluted 1 in 10. In order to test whether undiluted chylomicron plasma might exhibit some effect, activated frozen and thawed plasma samples from the top layer were substituted for activated substrate plasma. The results are shown in Table III. The accelerating effect of the chylomicron containing top layer can be explained exclusively by a effect of the platelets present. According the top layer of chylomicron-rich but plat let-poor plasma from a patient with seve thrombocytopenia had a much longer clotti time than the chylomicron-poor but mo platelet-rich top layer from the plasma of normal fasting person. A possible inhibito effect of high concentrations of chylomicro was excluded by the experiment presented Table IV, which shows that the recalcific tion time is independent of the concentrati of chylomicrons.

## DISCUSSION

It might be argued that the identificati and counting of blood platelets in chylomi ron suspensions is unreliable. However, t is not so when undiluted plasma is examin by the technique described. Dilution is n necessary, since red blood cells are not pr sent, and the platelet count is low. On stora

Table III. *The recalcification time activated samples from the top layer af high speed centrifugation.*

| Sample no. | Clotting time in sec. | Platelet number per mm³ in the undiluted top layer | Range of platelet numbers with same clotting activity |
|---|---|---|---|
| 1 | 54 | 4,150 | 3.100–4.700 |
| 2 | 90 | 620 | 530–740 |
| 3 | 239 | 30 | < 50 |

The samples were frozen and thawed twice, act ated, and then recalcified in the system descri in Table I. Sample 1 was taken after fasti Sample 2 was chylomicron-rich, taken after a fa meal. Sample 3 was taken from a patient w severe thrombocytopenia after a fatty meal, a was rich in chylomicrons.

ble IV. *Recalcification times of various tions of chylomicron-rich top layer pre- d by high speed centrifugation of plasma n a patient with severe thrombocytopenia (3,500 platelets per mm³ of blood).*

| | Per cent of chylomicron-rich top layer | | | | |
|---|---|---|---|---|---|
| | 100 | 50 | 25 | 10 | 0 |
| ting time ec | 239 | 249 | 255 | 258 | 265 |

sample was mixed in the given proportions a nearly platelet-free frozen and thawed nor- plasma. The mixed samples were activated, and recalcified in the system described in Table I.

at least 30 minutes in a moist chamber, platelets sediment to the bottom of the counting chamber and show the typical rphology, whereas the chylomicrons col- at the top and do not disturb platelet nting. Furthermore, chylomicrons have entirely different appearance, being small round, have no granules and give less trast than platelets. When dilution fluids h a low specific gravity are used, such as per cent ammonium oxalate (Brecher & nkite 1950), more chylomicrons sediment h the platelets in the counting chamber. ferentiation is still easy, but counting is re time-consuming. If dilution is desired, refore, a dilution fluid with a specific vity close to that of plasma should be sen, such as 3.13 per cent sodium citrate ydrate, which is now used in the method Nygaard (1933).

Why some of the blood platelets move to top during high-speed centrifugation, is known. The simplest explanation is that r specific gravity is slightly lower than that of plasma. Another possibility is that some platelets adhere to chylomicrons and are thus carried to the top. This theory is opposed by the fact that more platelets are present in the top layer after high-speed centrifugation of normal fasting plasma than of chylomicron-rich plasma.

The results presented suggest that the clot promoting effect of chylomicron suspensions is caused by the blood platelets present.

Intact platelets have no or very little effect in the clotting system. Poole (1955) examined the effect of chylomicron suspensions washed five times in saline. This procedure considerably increases the activity of the platelets present because of disintegration. Poole does not state the number of platelets present in the chylomicron suspensions before the washing procedure.

Kingsbury & Morgan (1957) carried out the washing and centrifugation of the chylomicron suspension in large amounts of distilled water. This procedure quite effectively causes platelet disintegration. Nothing is stated concerning the presence of platelets in the chylomicron suspensions. Our results suggest that blood platelets were present in the chylomicron suspensions studied by Poole and by Kingsbury & Morgan, and that platelets and not chylomicrons were responsible for the effect observed.

## SUMMARY

The activity of chylomicron suspensions in plasma was studied in a recalcification system employing activated, nearly platelet-free human plasma as a substrate. The chylomicron suspensions obtained from the top layer after high speed centrifugation of plasma contained a considerable number of plate-

lets. The clotting activity which has previously been ascribed to chylomicron suspensions is explained entirely by the platelets present. Other results also indicated that chylomicrons per se have no clot promoting activity.

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# THE *IN VITRO* STABILITY OF THE HAPTOGLOBIN-HEMOGLOBIN COMPLEX

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nvestigation of the biochemical nature of toglobin has furthered understanding of variations in the electrophoretic mobility nnell & Smithies, 1960), but the nature he haptoglobin-hemoglobin bond remains e clarified. The studies which we shall cribe were designed to test in a preli- ary manner the stability of the haptoglo- hemoglobin complex in a variety of *in o* environments.

## METHODS

emoglobin-binding capacity of haptoglobin ion was determined by the method of Jayle 1). Hemoglobin was quantitated as cyan- emoglobin (Crosby, Munn & Furth 1954). oactivity was measured in a well type scintilla- detector and sufficient counts were accumulated duce the counting error below 5 per cent.

*reparations of solutions:*

aptoglobin was obtained relatively free from proteins from ascitic fluid from patients with Type I haptoglobin by the method of Laurell (1959). The final concentration contained 8.7 to 41.9 mg of nitrogen/ml as haptoglobin. Radioiron-labeled hemoglobin was obtained from a 2 kg dog by the intravenous injection of 150 $\mu$C of $Fe^{59}$ as ferric chloride followed in 10 days by phlebotomy. The red cells were washed three times with 0.9 per cent sodium chloride and a hemoglobin solution prepared by the method of Hamilton, Farr, Hiller & Van Slyke (1947). This contained 47.3 mg/ml of hemoglobin and 2500 counts/minute/mg of hemoglobin. Non-radioactive hemoglobin was prepared in a similar manner from a normal dog. These solutions were stored at —16° C.

*B. Separation of haptoglobin-hemoglobin and unbound hemoglobin:*

Preliminary studies on diethylaminoethyl (DEAE) cellulose columns revealed that under the gradient elution system of Fahey, McCoy & Goulian (1958), free hemoglobin migrated with the initial gamma globulin peak whereas haptoglobin-hemoglobin was eluted between the peaks of beta globulin and albumin. Subsequent separations were carried out on column 36 x 1.8 cm packed under 0.7 kg/cm$^3$ air pressure. The samples to be separated were first dialyzed overnight against 0.01 M phosphate buffer at pH 8.0. 1 to 2 ml of test solution were placed on the top of the column following which development was carried out with 0.02 M

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Table I.

| $Fe^{59}$ Hemoglobin added to Haptoglobin | pH of Incubation mixture | Per cent Recovery | Per cent «Exchange» |
|---|---|---|---|
| First | 5.0 | 83 | 10 |
| Last | 5.0 | 84 | 9 |
| First | 7.1 | 62 | 2 |
| Last | 7.1 | 47 | 4 |
| First | 8.0 | 43 | 2 |
| First | 8.5 | 95 | 11 |
| Last | 8.5 | 87 | 14 |

Per cent recovery indicates proportion of radioactive hemoglobin initially subjected to separation which was found in the total amount recovered from the column.

Per cent exchange indicates the proportion of recovered radioactivity which appeared in the initially non-radioactive fraction.

phosphate buffer of pH 7.0 for unbound hemoglobin followed by 0.1 M sodium acid phosphate for elution of the haptoglobin-hemoglobin complex. Improvement in recovery was noted when the test solutions and developers were saturated with carbon monoxide prior to use. The concentration of the eluates was done by the method of Laurell (1958) followed by ultrafiltration (Mies, 1953).

*C. Experimental procedure:*

Radioiron-labeled hemoglobin was added to the haptoglobin solution in an amount calculated to be one-third to one-half of the solution's hemoglobin-binding capacity. After 15 minutes of incubation at 37° C, non-radioactive hemoglobin was added in an amount to exceed by 200 per cent the solution's hemoglobin-binding capacity. In a parallel experiment non-radioactive hemoglobin, was first added to the haptoglobin solutions in excess of the hemoglobin-binding capacity and incubation was then followed by the addition of radioiron hemoglobin in an amount similar to that used in the first experiment. Incubation was then carried out for two hours after adjustment of the pH to 7.1 or to 8.0 by dialysis against a phosphate buffer, to pH 8.5 with 3 per cent NaOH or pH 5.0 by dialysis against 0.02 M acetate buffer. The test solution was then returned to pH 8.0 with dialysis against phosphate buffer and placed on a DEAE column for separation.

## RESULTS

The observations are presented in Table I. Recovery is expressed in terms of the amount of radioactivity recovered in the concentrated eluate relative to the amount initially placed on the column. The degree of exchange is indicated by the per cent of the total activity found in the fraction recovered which contained only non-radioactive hemoglobin prior to the incubation. In no case did this exceed 14 per cent and in general was 10 per cent or less.

## DISCUSSION

These figures suggest that possibly some exchange occurs. It is conceivable that incomplete separation by the system may account for a simulated exchange. Another possibility is that during these experimental conditions no exchange occurs between a free and bound hemoglobin but rather between the heme in the bound and the heme in the free hemoglobin. This type of exchange is theoretically favored if the hemoglobin is partially oxidized to methemoglobin during chromatography. This was found to be the case when the hemoglobin bond was not stabilized by carbon monoxide.

*In vivo* saturation of plasma haptoglobin by intravenous injection of hemoglobin is followed by clearance of the entire haptoglobin-hemoglobin complex unassociated with detectable return of the haptoglobin through circulation (Franklin, Oratz, Rothschild & Zucker-Franklin 1960). However

ıer studies are necessary to determine lusively whether an exchange may occur *ivo* at a physiological pH between free haptoglobinbound hemoglobin.

## SUMMARY

he exchange of hemoglobin bound to oglobin with hemoglobin free in solution been studied *in vitro*. An exchange of than 15 per cent was observed following hours' incubation in the pH range 5.0 to The haptoglobin-hemoglobin bond would ear to be at least a fairly stable one under circumstances of this investigation.

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# DETERMINATION OF THE INSULIN-LIKE ACTIVITY IN SERUM USING RAT EPIDIDYMAL ADIPOSE TISSUE

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The publication of "An assay for insulin-like activity using rat adipose tissue" (Martin, Renold & Dagenais 1958) introduced a new and rather simple technique by which to determine the insulin-like activity in serum (hereinafter: SILA). As compared to the hitherto used rat diaphragm tissue assay the advantage of the new method lies in its much steeper dose-response curve. On the basis of the above, preliminary communication, the rat epididymal adipose tissue assay has been tested in a series of experiments in our laboratory where it has been modified and used in later studies of SILA in normal subjects and patients suffering from diabetes mellitus. Since the modified method differs slightly from the one suggested by Renold, Martin, Dagenais, Steinke, Nickerson & Sheps (1960) it shall be discussed in detail here and experiments to determine the precision of the method shall be described. Finally, experiments on the importance of various methods of preparing serum from whole blood are surveyed together with investi tions of the fluctuations in SILA af freezing and thawing of serum.

The specificity problems and the probl of recovery of added insulin will be discus in a later paper.

## PROCEDURE

The rat epididymal adipose tissue assay ta advantage of the two facts that incubated rat didymal adipose tissue is capable of convert 1-$C^{14}$ into $C^{14}O_2$, and that the rate of the proced is dependent on the insulin concentration in incubation medium. Specimens of adipose tissue incubated both in serum and in standard ins solutions upon which the $C^{14}O_2$ production is de mined; when the $C^{14}O_2$ production is known possible to calculate the insulin-like activity serum.

In the present modification of the rat epididy fat method, *standard insulin solutions* are prepa with a Krebs-Ringer bicarbonate buffer contai gelatin to which insulin and glucose are added. dilutions of freshly prepared insulin are used, insulin contents being 500 and 15.6 microunits ml ($\mu$U/ml), respectively. The buffer is comp as follows: $Na^+$ 144.1 meq/l, $K^+$ 4.7 meq/l, C 2.5 meq/l, $Mg^{++}$ 2.4 meq/l, $Cl^-$ 125.2 m $HCO_3^-$ 24.9 meq/l, $H_2PO_4^-$ 1.2 meq/l, $SO_4$

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1e I. *Distribution in incubation flasks of fat segments.*

| | Rat no. | | | |
|---|---|---|---|---|
| | I | II | III | IV |
| ard insulin tion 500 μU/ml | M | M | M | M |
| n I | D | P | D | P |
| n II | P | D | P | D |
| n III | D | P | D | P |
| n IV | P | D | P | D |
| ard insulin tion 15.6 μU/ml | M | M | M | M |

oximal) : Segment of fat tissue closest to the stis.

istal) : Segment of fat tissue remotest from ie testis.

iedial) : Segment of fat tissue from a site be- ween P and D.

eq/l, Gelatine (Merck) 200 mg/100 ml glucose ng/100 ml. The applied insulin preparation is f glucagon and produced from pig pancreas. nsulin content is 27.1 i.u./mg)[1].

diluted as well as diluted serum has been ned.

*um* is prepared from blood drawn by puncture slightly stased vein in the upper extremity; diately upon drawing, the sample is stored at for 30 to 60 minutes, and spun for 10 minutes 0 r.p.m. at room temperature. The coagulum ached by a wooden stick and re-spun for 5 s at 4000 r.p.m. Serum is pipetted off and at 4° C. until incubation, 4 to 6 hours after ood was drawn. The glucose concentration in is determined by the glucose oxidase method ett & Nixon 1957).

*diluted serum* is prepared by mixing one of serum and four batches of Krebs-Ringer nate buffer containing gelatin. Serum and dilutions are adjusted to the same glucose tration as the standard insulin solutions by

pplied through the courtesy of Dr. H. C. rn, M.D. Nordisk Insulin Laboratorium.

addition of glucose in medium. 0.1 ml of glucose-1-$C^{14}$ solution containing 0.05 μCurie and 1.9 ml of serum, serum dilution, or standard insulin solution is filled into incubation flasks (Fig. 1) with total capacities of 55 ml.

Four male rats of the Wistar strain, weighing 130—200 g are used in each experiment; the animals are taken directly from the cages where they have been feeding *ad libitum;* they are killed by a blow on the head and decapitated. After exsanguination the abdomen is opened and the epididymal tissue gently taken up. Each of the two epididymal fat pads is cut into three segments and dropped into their appropriate incubation flasks according to a distribution shown in Table I, *viz.* tissue segments cut medially in the fat pad are dropped into all flasks containing standard insulin solution, whereas two tissue segments cut proximally, and 2 cut distally in the fat pad are incubated in serum.

The incubation flasks are weighed before and after addition of the segments of fat tissue. Weights of segments thus determined range between 30—200 mg. The flasks are fitted with stoppers provided with two ports and glass tubing with rubber fittings (Fig. 1). Small stainless steel cups, with internal diameters of 13 mm, are suspended below the stoppers. Discs of lens paper cover the bottoms of the cups. Later these cups

Fig. 2. *The incubation flask.*

serve as counting cups in a proportional flow counter.

Before incubation the flasks are gassed with 95 per cent of $O_2 + 5$ per cent of $CO_2$ for 5 minutes using thin needles inserted through the rubber fittings. Subsequently they are incubated for 2 hours at 37° C in a Dubnoff metabolic agitator (Dubnoff 1948) using 86 shaking cycles per minute: the stroke has a length of 5 cm. After incubation 0.2 ml of 10 N $H_2SO_4$ is introduced into the incubation medium by means of thin needles and 0.5 ml of a 2/19 saturated NaHO solution is introduced into the steel cups. If 0.2 ml $H_2SO_4$ causes coagulation of the incubation medium a further 0.8 ml $H_2SO_4$ is added. After storage for 2 hours the cups are taken out and dried at 37° C for 8—12 hours, later at 60° C for 2 hours and finally at 160° C for 1 hour. Weights of contents in the steel cups range between 13 and 15 mg. When cooled the cups are transferred to a proportional flow counter (Friesecke & Hoepfner FH 407); the total number of counts per sample has been 1000 x 2. The activity of the weakest sample corresponds to about 100 counts per minute (hereinafter: CpM). The background radiation of the flow counter is 15—20 CpM.

## *Determination of SILA and Index of precision.*

The insulin-like activity of the incubated sera is determined as follows: The $C^{14}O_2$ production in the incubation flasks is expressed as: log Ak = log CpM x 10: $\sqrt{\text{fat weight}}$, CpM here denoting the rate of counts per minute as registered from the counting cup without correction for self absorption, the fat weight being "wet weight". As to the segments of adipose tissue incubated in standard insulin solutions with 500 μU/ml, the average is calculated of the 4 log Ak values, m log Ak (500). Calculations were made of the corresponding arithmetic mean of tissue segments incubated in standard insulin solutions with 15.6 μU/ml (m log Ak (15.6)), and in serum (m log Ak(s)). The dose-response line is plotted on a co-ordinate system in which log As is the ordinate and the logarithm to the insulin concentration is the abscissa, by plotting m log Ak (500) and m log Ak (15.6) and connecting the two points by a straight line. Subsequently the insulin-like activity of a serum is determined by interpolation of the corresponding m log Ak(s) value on this line. SILA values thus determined ranging below 10 μU/ml are recorded as 0 μU/ml.

The steepness of the dose response curve of adipose tissue segments from different rats varies individually. The steepness is calculated as

$$b = \frac{\log Ak\ (500) - \log Ak\ (15.6)}{\log 500 - \log 15.6}$$

If the b-value of the dose response curve of adipose tissue segments from a rat in a certain experiment is under 0.2, the corresponding log Ak values have been left out in the calculation of m log Ak. If more than one rat in an experiment has b-values below 0.2 such an experiment is invalidated.

The precision index is determined by the expression s:b, b denoting the steepness of the dose response curve, s representing the "rest standard deviation" of the log Ak values, *viz.* the standard deviation obtained in an experiment after effects of the differences between rats and effects of differences between incubation media have been removed. However, the rest standard deviation has not been corrected for such variations as are ascribable to differences of the segments cut from proximal and distal sites in the fat pad. Such correction is impracticable because the medial segments in the present work always are incubated in standard insulin solutions. Otherwise the precision index is determined as suggested by Sheps, Nickerson, Dagenais, Steinke & Martin (1960).

## *Precision of the method for the recovery of $C^{14}O_2$.*

The technique used in the above discussed modified rat epididymal fat method for the recovery determination of the $C^{14}O_2$ production has been tested in some experiments. In 24 incubation flasks is pipetted off 0.1 ml of a solution containing $Na_2C^{14}O_3$ and 1.9 ml of 25 mM $Na_2CO_3$ solution. The flasks are stoppered and gassed with a $O_2 + CO_2$ mixture upon which the $C^{14}O_2$ production is recovered and determined as registered in the experiment instructions.

*ights and distribution of the fat segments he epididymal fat pads.*

he interrelation of the weight of the segment the $C^{14}O_2$ production is examined; 12 incuba- flasks contain standard insulin solutions with μU/ml; 12 other flasks standard insulin solu- with 500 μU/ml.

ne of the fat pads from a rat is cut into 3 seg- ts (a proximal, a distal, and a medial segment); segments are dropped into 3 incubation flasks aining 500 μU/ml. Likewise the other fat pad t into 3 segments and dropped into flasks con- ng 15.6 μU/ml. Four rats are used in each riment. The fat pads from rats Nos. 1 and 2 cut as to make the distal segment small, the ial segment a little larger, and the proximal ent the largest. The fat pads from rats Nos. 3 4 are cut so as to make the distal segment e, the medial segment a little smaller, and the imal segment smallest. All segments are in- ted and the $C^{14}O_2$ production determined as stered in the instructions.

*ulin concentration and $C^{14}O_2$ production.*

ne interrelation of the logarithm of the insulin entration and the logarithm of the $C^{14}O_2$, ex- sed as $\log Ak = \log CpM \times 10 : \sqrt{\text{fat weight}}$ lustrated in experiments in which segments of issue are incubated in standard insulin solutions 500, 250, 125, 62.5, 31.3, and 15.6 μU/ml. The edure explained in the instructions is used, and ents are distributed as shown in Table I, ex- that serum I is replaced by a standard insulin ion containing 250 μU/ml and serum II is re- d by a standard insulin solution containing μU/ml, *etc.*

*hod for the separation and ing of serum.*

ood drawn by venipuncture from fasting nor- subjects is stored either at 4° C or at room erature (18—20° C). Serum is prepared from that has been stored at 4° C (cf. instruc-). Blood is stored at room temperature for rs before spinning for 10 minutes at 4000 r.p.m. coagulum is detached by a wooden stick be- fore re-spinning at 4000 r.p.m. for 5 minutes: finally the serum is pipetted off.

A batch of serum prepared under these different temperature is stored at 4° C after centrifugation and SILA is determined the same day. The re- mainder is exposed to —18° C. SILA in the frozen serum is determined after intervals of 1 or 6 weeks. The thawing process takes place at room temperature for 30 minutes immediately upon which the serum is stored at 4° C until incubated.

## RESULTS

The precision of the technique for the re- covery and determination of the $C^{14}O_2$ pro- duction is tested in 8 experiments; identical volumes of $Na_2C^{14}O_3$ are pipetted off into 24 incubation flasks in all of the experiments. The coefficient of variation of rates of CpM registered per counting cup is determined. The average of the coefficient was found to be: 4.45 per cent, the highest registration being 7.8 per cent, the lowest 3.4 per cent.

Fig. 2 illustrates that a pipetting off of different quantities of $Na_2C^{14}O_3$ into incuba- tion flasks makes it possible to demonstrate a rectilinear interdependence of $C^{14}O_2$ pro- duction and rate of CpM registered per counting cup.

Findings from studies of the influence of weights of segments on the $C^{14}O_2$ production are registered in Table II. The table records the $C^{14}O_2$ production in segment of varying sizes incubated in 500 and 15.6 μU/ml; from the experimental disposition it appears that large, medium, and small tissue segments originate from the same rat. The $C^{14}O_2$ pro- duction in one segment is determined by two formulas, *viz.* (1) as:

$$Ak = CpM \times 10 : \sqrt{\text{fat weight}}$$

and (2) as: $A = CpM \times 100 :$ fat weight. From Table II it will be noted that if the

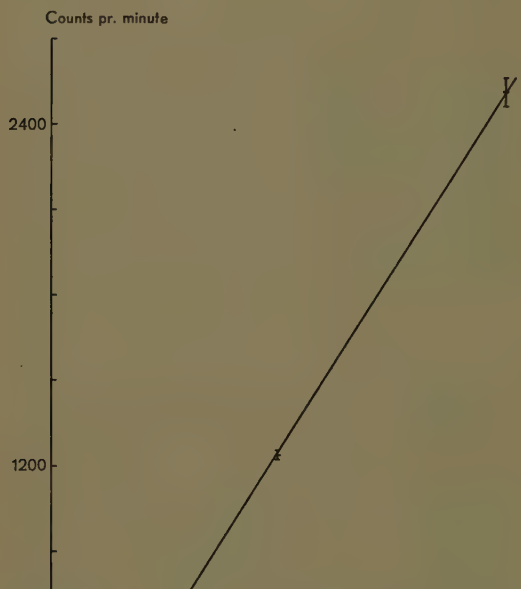



Fig. 2. The interdependence of the volume of $Na_2C^{14}O_3$ in the incubation flasks and the number of CpM registered per counting cup.

$C^{14}O_2$ production is determined as A, values will be higher for small than for large segments in either insulin concentration. The difference between the average values of small and large tissue segments is statistically significant (paired data t test, $p < 0.01$) at the insulin concentration 500 $\mu$U/ml, but is not statistically significant ($0.1 > p > 0.05$) at 15.6 $\mu$U/ml. If $C^{14}O_2$ is calculated as Ak, the average values of large segments will be found to be higher than those of small se ments in either insulin concentration, b this difference is not statistically significa ($p > 0.1$).

It will be noted from column 5 in Table that small, medium, and large segments adipose tissue have been taken from dist medial, and proximal parts of the fat pad. either of the examined insulin concentratio the average Ak values of proximal segmen were found to be 14—16 per cent lower tha values of medial segment in contrast values of distal segments which were high by 10—15 per cent. However, the differen are not statistically significant ($p > 0.1$).

The interrelation of insulin concentrati and $C^{14}O_2$ production is illustrated by fo experiments. These, carried out in qua ruplicate, served to demonstrate the inte relation of the $C^{14}O_2$ production (calculat as $\log Ak = CpM \times 10 : \sqrt{\text{fat weight}}$) a the logarithm to the insulin concentrati (log I) within the area: 15.6 to 500 $\mu$U/m It will be noted from Figs. 3, 4, 5, and 6 th a rectilinear interdependence is demonstrat between log Ak and log I in this area of co centration (Figs. 3, 4, 5, and 6).

The index of precision (lambda) of th modified rat epididymal fat method is c culated on the basis of serial determinatio of the insulin-like activity of serum, carri out in a period from December 1959 to N vember 1960. A total of 138 examinatio were carried out. The index of precision the triplicate experiments (*cf.* Table II fluctuated around an average of 0.30. T quadruplicate examinations showed avera values of 0.28.

The influence of temperatures at wh serum is prepared was examined in 10 n

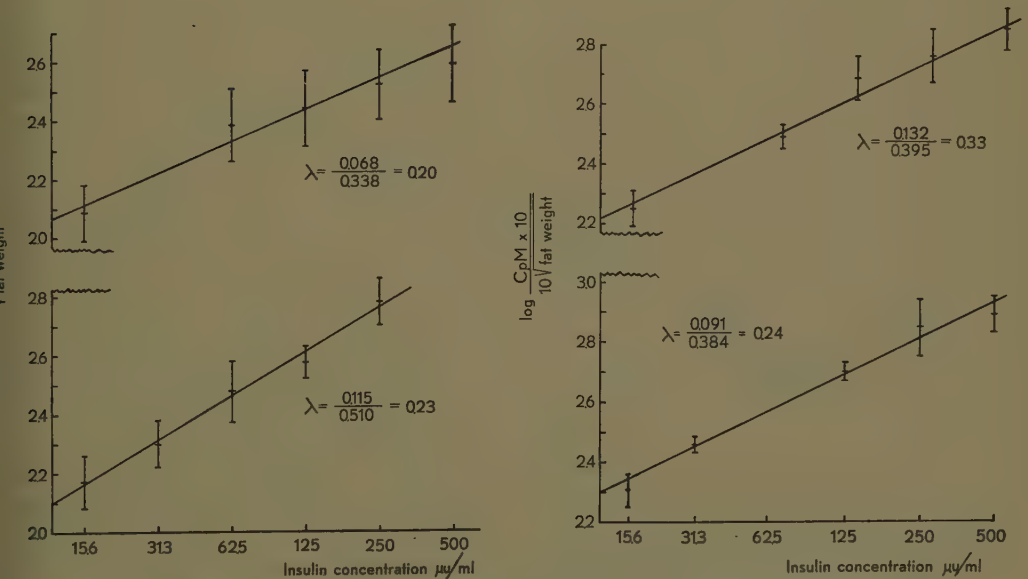
Table II. *The interrelation of the weight of the fat segment and the $C^{14}O_2$ production from glucose-1-$C^{14}$.*

| ze of nents | Insulin conc. µU/ml | Nos. of segments | Weight mg | Mean weight mg | Distribution of segments | | | $A = \frac{CpM \times 100}{\text{fat weight}}$ meanfig. ± standard deviation | $A = \frac{CpM \times 10}{\sqrt{\text{fat weight}}}$ meanfig. ± standard deviation |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | D | M | P | | |
| ll | 500 | 15 | 20– 93 | 53.3 | 8 | | 7 | 10580±6100 (a.1) | 7130±3340(a.2) |
| ium | 500 | 15 | 37–114 | 88.0 | | 15 | | 9002±5520 (b.1) | 7861±3980(b.2) |
| e | 500 | 15 | 71–209 | 140.3 | 7 | | 8 | 7304±3750 (c.1) | 7694±3890(c.2) |
| ll | 15.6 | 13 | 20–107 | 55.8 | 6 | | 7 | 3056±1490 (d.1) | 2096±1560(d.2) |
| ium | 15.6 | 13 | 43–166 | 92.9 | | 13 | | 2641±1470 (e.1) | 2295±1020(e.2) |
| e | 15.6 | 13 | 74–196 | 132.4 | 7 | | 6 | 2241±1350 (f.1) | 2461±1280 (f.2) |

.1 ~ c.1 $t = 3.445$ $p < 0.01$
.1 ~ f.1 $t = 1.737$ $0.1 > p > 0.05$

a.2 ~ b.2 $t = 1.029$ $p > 0.1$
a.2 ~ c.2 $t = 0.820$ $p > 0.1$
d.2 ~ f.2 $t = 0.983$ $p > 0.1$

D = distal fat segment. M = medial fat segment. P = proximal fat segment.




Figs. 3, 4, 5, and 6. The effect of Insulin on the $C^{14}O_2$ production from glucose 1 $C^{14}$.

Table IV. *The importance of using different methods for the production and storage of serum.*

| Subject no. | SILA determined the day when blood was drawn: No. of rats | Index of precision | SILA in blood stored at 4° C.: undiluted | SILA in blood stored at 4° C.: diluted | SILA in blood stored at room temperature: undiluted | SILA in blood stored at room temperature: diluted | SILA determined after freezing and thawing of serum: No. of rats | Index of precision | SILA in blood stored at 4° C.: undiluted | SILA in blood stored at 4° C.: diluted | SILA in blood stored at room temperature: undiluted | SILA in blood stored at room temperature: diluted |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 4 | 0.33 | 120 | 90 | 280 | 435 | 4 | 0.18 | 198 | 160 | 188 | 185 |
| 2 | 4 | 0.21 | 182 | 435 | 335 | 250 | 3 | 0.27 | 132 | 425 | 430 | 460 |
| 3 | 4 | 0.27 | 42 | 0 | 25 | 0 | 4 | 0.23 | 200 | 635 | 350 | 625 |
| 4 | 3 | 0.33 | 150 | 200 | 182 | 150 | 4 | 0.13 | 73 | 275 | 90 | 250 |
| 5 | 4 | 0.30 | 105 | 195 | 170 | 220 | 4 | 0.13 | 175 | 485 | 165 | 525 |
| 6 | 3 | 0.39 | 90 | 128 | 130 | 130 | 3 | 0.15 | 245 | 360 | 238 | 430 |
| 7 | 3 | 0.23 | 110 | 125 | 118 | 240 | 4 | 0.22 | 150 | 165 | 175 | 160 |
| 8 | 4 | 0.30 | 46 | 125 | 115 | 230 | 4 | 0.30 | 140 | 165 | 103 | 175 |
| 9 | 3 | 0.24 | 66 | 90 | 102 | 165 | 4 | 0.25 | 132 | 220 | 280 | 240 |
| 10 | 3 | 0.40 | 98 | 0 | 190 | 0 | 4 | 0.30 | 132 | 305 | 310 | 350 |
| mean * | | 0.30 | 100.9 A | 138.8 B | 164.7 C | 182.0 D | | 0.22 | 157.7 E | 319.5 F | 232.9 G | 340.0 H |

A ~ C $t = 3{,}505$ $p < 0{,}01$ B ~ D $t = 1{,}002$ $p > 0{,}1$ C ~ G $t = 1{,}692$ $p > 0{,}1$
A ~ E $t = 2{,}367$ $p < 0{,}05$ B ~ F $t = 2{,}948$ $p < 0{,}02$ D ~ H $t = 1{,}971$ $p < 0{,}1$
A ~ G $t = 3{,}713$ $p < 0{,}01$ B ~ H $t = 3{,}239$ $p < 0{,}02$

Table III. *Index of Precision.*

| Mean ± stadard error | Triple assays | Quadruple assa |
|---|---|---|
| No. of assays | 48 | 90 |
| s | 0.105 ± 0.004 | 0.103 ± 0.003 |
| b | 0,351 ± 0.016 | 0.369 ± 0.007 |
| lambda | 0.297 ± 0.013 | 0.279 ± 0.009 |

mal subjects (Table IV). It was demo strated that SILA values in undiluted seru prepared from blood stored at 4° C we lower than values in undiluted serum pr pared from blood stored at room temper ture. Average figures from the two seru groups are: 100.9 μU/ml and 164.7 μU/m respectively; the difference between the two averages is statistically significa (paired data t test, $p < 0.01$). The arithmet mean of SILA determined in undilut serum is also higher in serum prepared 4° C than in serum prepared at room te perature, but the difference is not statisti ally significant ($p > 0.1$).

The influence of freezing on serum's ins lin-like activity is recorded in Table IV. T average SILA value before and after exp sure to cold in undiluted serum stored 4° C is 100.9 and 157.7 μU/ml, respectivel the difference is statistically significa ($p < 0.05$). Corresponding averages in d diluted serum are 138.8 and 319.3 μU/m and also this difference is statistically sig ficant ($p < 0.02$). Hence, increased SIL values are found after freezing and thawi in undiluted as well as in diluted sera.

Findings seem to be similar in serum pr pared at room temperature. The avera SILA values in undiluted and diluted se were found to be higher after freezing, b the difference is not statistically significa

It is hardly surprising that differences are st pronounced if SILA values in serum pared at 4° C and examined before freez- are compared with SILA values in um prepared at room temperature and mined after freezing. The latter values significantly higher in undiluted as well in diluted serum (Table IV).

## DISCUSSION

The technique here discussed for the re- very and determination of $C^{14}O_2$ produc- n is more convenient than the hitherto d assay by which $C^{14}O_2$ was recovered in OH and settled by $BaCl_2$. The method is s time-consuming than the one suggested Renold *et al.* (1960) and calculations are re simple because no correction for self sorption is required.

Renold *et al.* (1960) stated earlier that if $C^{14}O_2$ production in segments of adipose sue are calculated as: CpM $\times$ 100: fat ight values will be higher in segments ighing less than 70 mg than in larger seg- nts. Naturally, segments of identical ights are not easily obtained and hence it ght prove adequate if the SILA deter- nation were corrected for such fluctua- ns on fat weights. Such correction has en practicable in the present work *viz.* by culation of the $C^{14}O_2$ production as: M $\times$ 10: $\sqrt{\text{fat weight}}$.

Renold (cf. above) found production of $^4O_2$ to be significantly lower in proximal ments than in medial and distal segments. the present material, values from proxi- l segments seemed to be lower. Values of distal segments were higher whereas ues of the medial segments corresponded the average values of proximal and distal segments. This feature was seen both at high and low insulin concentrations although the difference was not statistically significant.

With due regard to these facts a modification of the method suggested by Renold has been used in subsequent studies of SILA values in normal subjects and in diabetics. The modification differs from the original method in four respects *viz.* (1) proximal, distal and medial segments are distributed so as to neutralize the differences in $C^{14}O_2$ production (Table I); (2) four rats have been used in each experiment; (3) $C^{14}O_2$ is picked up directly in the counting cups; and (4) the $C^{14}O_2$ production is calculated as

$$\text{CpM} \times 10 : \sqrt{\text{fat weight}}.$$

By this modification of the rat epididymal fat method, values of the index of precision have been found to correspond with values obtained by the original method (Sheps *et al.* 1960). The latter authors obtained the most adequate lambda values, *i. e.* the lowest values, in experiments in which the dose response curves were steepest, which is in accordance with findings from the present study. Hence it is preferable, with a view to the precision of the experiments, to eliminate animals presenting flattened dose response curves before the SILA values are determined. Since the modified method used in the present work include four rats in each experiment it has been possible to reject a rat if its dose response was found unsuitable and still retain a threefold determination of serum.

Martin *et al.* (1958) found insulin concentration of 10 $\mu$U/ml to be the lowest concentrations demonstrable by the rat epididymal adipose tissue assay; in conformity herewith all SILA values ranging below

10 μU/ml are here recorded as: 0 μU/ml.

It remains to be explained why there is a difference between serum prepared at 4° C and serum prepared at room temperature; it is hardly probable that insulin decomposes at 4° C. An insulin antagonist may well be decomposed at room temperature but the problem has not been investigated. And it is still unknown whether insulin, inactively bound to protein, may be liberated at room temperature. Protein bound insulin has been found to accelerate the glucose metabolism in adipose tissue (Gundersen & Anthoniades 1960) but detailed, quantitative information of the effects of liberated and bound insulin on such tissue is not available.

Serum prepared at 4° C is assumedly the best criterion of SILA, *viz.* the total content of insulin and insulin-like substances together with the content of insulin antagonists, at the time of blood sampling. Hence, this technique has been used for the preparation of serum in later examinations.

In the present work increased SILA values were demonstrable when serum was prepared at room temperature and frozen but once. This is in harmony with findings from our laboratory where chilled, fresh serum has been used. Normal values were found to range lower (Lyngsøe 1962) than normal values reported by Sheps *et al.* (1960).

## SUMMARY

A modification of the rat epididymal fat method for the determination of the insulin-like activity of serum is described. The modified method has been found to be more convenient than the original assay, and the precision of the method, determined by the index of precision, is of the same order.

The insulin activity of serum has been found to be dependent on the technique by which serum is prepared. Cold preparation of fresh serum will give the lowest values.

## ACKNOWLEDGEMENTS

I wish to express my gratitude to Miss Tove Dreyer Petersen for skilled technical assistance.

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# A SIMPLIFIED METHOD FOR THE DETERMINATION OF XYLOSE IN URINE

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The method for determination of xylose in ne according to Roe & Rice (1948) in- des the laborious step of Somogyi pre- itation of proteins and chromogens. When the method is used for the deter- nation of xylose in urine in the xylose test enson, Culver, Ragland, Jones, Drum- y & Bongas 1957) this step can be ex- ded without invalidating the accuracy of method. Further, the incorporation of a nk for each urine sample, as in the origi- method, can also be excluded as will be wn below.

## METHOD

*simplified method*

pparatus: Water-bath, thermoregulated C ± 1° C. Spectrophotometer Beckman B, 1 cm s, or similar spectrophotometer.

eagents: 1) P-bromoaniline (Eastman lak company) 2) Glacial acetic acid (p. a. ck, sp.g. 1.055—1.059) 3) Thiourea (Thioharn- f, Merck) 4) Xylose purum (Schuckhardt) and Bezoic acid (p. a. Merck).

olutions: P-bromoaniline reagent and se standard are prepared according to Roe & 1948.

*cedure*

efore the determination of xylose the urine ld be tested for glucose by a specific test *e. g.* with glucose oxydase paper. If glucose is present, a quantitatiive determination has to be done. If it does not exceed 4 per cent the glucose will not seriously interfere with the determination. If the glucose concentration is higher than 4 per cent the xylose can not be determined with accuracy even if a correction factor is introduced. *Vide infra.*

*Dilution of urine*

Two dilutions of each urine sample are always prepared in order to obtain absorbancies within the optimal range for the reading.

A) Urine volume above 400 ml (in five hours): Dilute 2 ml and 4 ml respectively to 200 ml with distilled water.

B) Urine volume less than 400 ml (in five hours): Dilute 1 ml and 2 ml respectively to 200 ml with distilled water.

*Determination of xylose*

Adjust the waterbath to 70 ± 1° C. Add 1 ml of the working standard to three test tubes and 1 ml of the two urine dilutions to two tubes. To all five tubes add 5 ml of the p-bromoaniline reagent, mix well. Place one of the tubes containing standard solution in the dark (as a blank) and the other tubes immediately in the waterbath for ten minutes. Then cool in tap water to room temperature and place the tubes in the dark for 70 minutes. The absorbancies are read against the blank in 1 cm cells at 520 mμ in a Beckman B spectrophotometer.

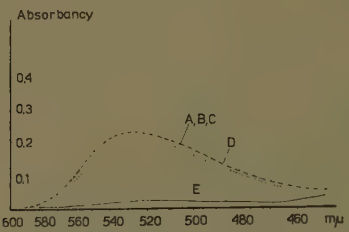


Fig. 1. Absorbancy curves of xylose in water and urine with and without the presence of protein and glucose. A Zeiss spectrophotometer model RPQ 20 A was used. In curves B, C, D the urines were diluted 1:50. All samples except "E" contained xylose (0.1 mg/ml) in the final dilutions.

Curve A: A solution containing 0.1 mg/ml xylose in water.

Curve B: A urine containing no protein and no glucose.

Curve C: A urine containing 6 per mille protein.

Curve D: A urine containing 4 per cent glucose.

Curve E: A solution of 4 per cent glucose diluted 1:50.

*Calculations*

The amount of xylose excreted in 5 hours is calculated according to the formula:

$$\text{g xylose} = \frac{0.1 \times \text{urine dilution factor} \times \text{ml urine}}{1000} \times \frac{\text{absorbance unknown}}{\text{absorbance standard}}$$

Readings within the range 0.075—0.400 should be used for the calculations.

## EXPERIMENTAL

1. *Absorbancy curves of xylose in water and urine with and without the presence of protein and glucose respectively.*

Xylose was dissolved in water and in three different urines, one free from protein and glucose, one containing 6 per mille protein and one containing 4 per cent glucose. The final concentration of xylose after dilution of the urines 1:50 was 0.1 mg/ml. The color development was accomplished according the technique described and the absorban at different wave lengths between 600 a 450 mμ was measured.

The absorbancy curves corresponding xylose in water and urine respectively wi and without protein coincide whereas t curve for the urine containing glucose sho a somewhat higher absorbancy but has t same shape. The absorbancy curve from 4 per cent glucose solution in water, dilut 1:50, shows a slight absorbancy at the di erent wave lengths.

It is evident from this experiment that protein concentration of 6 per mille and t chromogens in the urines in question wh diluted 1:50 did not influence the absorban of xylose as compared with xylose conce trations and in different urines will be d monstrated in the following experiments.

2. *Recovery of xylose added to protein- a glucose-free urines.*

To 8 different urines were added differe amounts of xylose according to Table I. T urines were diluted 1:50. In this experime blanks were made for each urine sample well as for the standard as described in t original method in order to register t variations of the different urine blanks. absorbancies for the blanks as well as the heated tubes were read against distill water. The amount of xylose was calculat by using the blank for the standard soluti as proposed for the routine technique.

The recovery varied from 100 to 105 p cent (mean 102.3 per cent) when using t standard blank for the correction and fr 100 to105 per cent (mean 102.9 per ce when using the individual blanks.

ble I. *Recovery of xylose added to pro- and glucose-free urines. The final urine tion was 1:50. The calculations were made by using the standard blank.*

| sample No. | Amount xylose added mg/ml | Absorbancy of blanks | Absorbancy of sample | Amount xylose found mg/ml | Recovery per cent |
|---|---|---|---|---|---|
| 1 | 0 | 0.010 | 0.016 | | |
| 2 | 0.040 | 0.004 | 0.096<br>0.096 | 0.0395 | 100 |
| 3 | 0.060 | 0.005 | 0.141<br>0.141 | 0.060 | 100 |
| 4 | 0.080 | 0.009 | 0.196<br>0.190 | 0.083 | 104 |
| 5 | 0.100 | 0.008 | 0.231<br>0.237 | 0.100 | 100 |
| 6 | 0.120 | 0.010 | 0.282<br>0.290 | 0.124 | 103 |
| 7 | 0.140 | 0.010 | 0.336<br>0.338 | 0.147 | 104 |
| 8 | 0.160 | 0.010 | 0.385 | 0.168 | 105 |
| 0.1 mg/ml | | 0.008 | 0.232<br>0.230 | | |

s seen from Table I, the blanks for the erent urines vary very little from the k for the standard solution and the in- dual urine blanks may therefore be ted for routine determinations as pro- d. With the dilutions of 1:100 and 0 the errors will be still smaller.

*Recovery of xylose added to different rines containing protein and glucose.*

ylose was added to different urines con- ng different concentrations of protein n 0 to 6 per mille) and to urine con- ng from 0 to 3.9 per cent glucose. The dilutions were 1:200.

As will be seen from Table II, the recoveries vary between 100 and 105 per cent, thus demonstrating that protein and glucose within the concentrations used are without interference on the recovery of xylose.

4. *The influence of different dilutions of the urine samples on the recovery of xylose added.*

In order to investigate the necessary dilutions of the urines in order to avoid the influence of chromogens, protein, and glucose at rather high concentrations; different urines containing these substances were tested at different dilutions. A urine of normal yellow color was diluted from 1:5 to 1:200 and xylose was added to a concentration of 0.1 mg/ml in the final solution and analyzed according to the routine technique. Two other urines, one containing 8 per cent glucose and one containing 6 per mille protein were diluted and analyzed in the same way.

As seen from Table III (samples 1-5),

Table II. *Recovery of xylose from urines containing protein and glucose. The final urine dilution was 1:200.*

| Urine sample No. | Glucose per cent | Protein per mille | Amount xylose added mg/ml | Amount xylose found mg/ml | Recovery per cent |
|---|---|---|---|---|---|
| 1 | 2.1 | | 0 | | |
| 2 | 0.1 | | 0.025 | 0.025 | 100 |
| 3 | 3.9 | | 0.050 | 0.051 | 102 |
| 4 | 0.3 | | 0.100 | 0.104 | 104 |
| 5 | | 0.4 | 0 | | |
| 6 | | 1.0 | 0.025 | 0.0225 | 102 |
| 7 | | 1.5 | 0.050 | 0.0525 | 105 |
| 8 | | 1.0 | 0.100 | 0.103 | 105 |

Table III. *Recovery of xylose added in varying concentration to different urines. Samples 1 to 6 contained no protein or glucose, samples 7 to 12 contained 6 per mille protein but no glucose, and samples 13 to 18 8 per cent glucose but no protein. Xylose was added to all samples to a concentration of 0.1 mg/ml in the final dilution.*

| Urine sample No. | Dilution of urine | Amount xylose found mg/ml | Recovery of xylose per cent |
|---|---|---|---|
| 1 | 1:200 | 0.099 | 99 |
| 2 | 1:100 | 0.0985 | 99 |
| 3 | 1:50 | 0.100 | 100 |
| 4 | 1:25 | 0.102 | 102 |
| 5 | 1:10 | 0.104 | 104 |
| 6 | 1:5 | 0.112 | 112 |
| 7 | 1:200 | 0.100 | 100 |
| 8 | 1:100 | 0.100 | 100 |
| 9 | 1:50 | 0.105 | 105 |
| 10 | 1:25 | 0.105 | 105 |
| 11 | 1:10 | 0.103 | 103 |
| 12 | 1:5 | 0.104 | 104 |
| 13 | 1:200 | 0.108 | 108 |
| 14 | 1:100 | 0.103 | 103 |
| 15 | 1:50 | 0.128 | 128 |
| 16 | 1:25 | 0.136 | 136 |
| 17 | 1:10 | 0.174 | 174 |
| 18 | 1:5 | 0.230 | 230 |

the recoveries are within the limits of those found in the former experiments (Tables I and II) at dilutions from 1 : 10 to 1 : 200. At a dilution of 1 : 5 (sample 6) the recovery is too high (112 per cent), demonstrating the interferance of some other substances in the urine. It is assumed that these substances were not of chromogenic origin since the absorbancy value for the blank was not raised. In samples 7-12 (dilutions of the urine containing 6 per mille protein) the recovery varied between 100 and 105 per cent indicating no influence of protein even at a dilution of 1 : 5. Thus a urine dilution of 1 : 50, which is the lowest dilution used in the routine technique, should make allo ance for a protein concentration of 6 per c in the undiluted urine.

When analysing the urine containing 8 cent glucose (samples 13-18 in Table I there was an increase (128 per cent) in recovery at a dilution of 1 : 50 but not 1 : 100. Therefore the highest concentrati of glucose tolerated in the undiluted urine 4 per cent using a dilution of 1 : 50. An eli nation of higher concentrations of glucose well as lower ones by the use of gluc oxidase does not seem to be possible as it found that xylose decreases in urine kept 37° C for a couple of hours even without

Table IV. *Excretion of xylose in uri from 11 normal individuals after intake 25 g xylose according to Benson et al. 19*

| Case No. | Sex | Age | Urine volume in 5 hours | Urine dilution | Amou xylos excrete 5 hou |
|---|---|---|---|---|---|
| | | | | | gm |
| 1 | ♂ | 32 | 330 | 1:100<br>1:200 | 6.9 |
| 2 | ♀ | 43 | 870 | 1:50<br>1:100 | 6.3 |
| 3 | ♂ | 37 | 1175 | 1:50<br>1:100 | 8.0 |
| 4 | ♀ | 45 | 950 | 1:50<br>1:100 | 7.0 |
| 5 | ♀ | 41 | 450 | 1:50<br>1:100 | 5.1 |
| 6 | ♀ | 70 | 820 | 1:50<br>1:100 | 5.5 |
| 7 | ♀ | 38 | 1000 | 1:50<br>1:100 | 6.9 |
| 8 | ♂ | 45 | 375 | 1:100<br>1:200 | 9.3 |
| 9 | ♂ | 76 | 575 | 1:50<br>1:50 | 5.5 |
| 10 | ♀ | 72 | 1000 | 1:50<br>1:100 | 6.3 |
| 11 | ♀ | 41 | 1000 | 1:50<br>1:100 | 6.1 |

ence of glucose oxidase. When the urine stored at room temperature for 72 hours xylose was found to disappear nearly pletely. When the urine was stored in ce box (+ 4° C) there were slight or hanges in concentration after 72 hours.

*Excretion of xylose in 11 normal individuals after intake of 25 gm xylose according to Benson (2).*

he prescriptions of Benson were strictly wed and the xylose in urine was analyzed rding to the simplified method. The re- are given in Table IV. The 5-hour ex- on varied between 5.1 and 9.3 g and is in the limits of 5-8 g as given by Benson the exeption of case No. 8 who ex- ed 9.3 g.

## SUMMARY

The method of Roe & Rice (1948) for determination of pentoses in biological erial as applied for the determination of se in urine has been reinvestigated.

2. The elimination of protein from the urine was not found necessary when the urines were diluted 1:50 to 1:200 and the amount of protein in the undiluted urine did not exceed 6 per cent.

3. Correction for chromogens by use of individual urine blanks was not found necessary since readings against the standard blank gave correct values.

4. A concentration of 4 per cent glucose in the undiluted urine did not interfere with the determination of xylose.

5. By omitting the protein precipitation and individual urine blanks as described in the original method of Roe & Rice (1948), the technique has been highly simplified.

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# THE AMOUNT OF TRAPPED PLASMA IN A HIGH SPEE MICRO-CAPILLARY HEMATOCRIT CENTRIFUGE

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The relative volume of red cells in a blood sample is a quantity of fundamental importance in clinical hematology and other fields of research. It is usually estimated as the relative volume of the packed red cell column after centrifugation of the blood sample. For very accurate work, the amount of plasma in the packed red cell column must be measured or a suitable correction factor applied.

Chaplin & Mollison (1952) made a thorough investigation of the amount of trapped plasma obtained when blood was centrifuged according to the Wintrobe (or similar) technique. The correction factors given by Chaplin & Mollison should apply to all methods using similar centrifuging conditions, *i. e.* a relative centrifugal force of about 1500 g and a duration of centrifugation of between 30 and 55 minutes. It is evident from the data of Chaplin & Mollison that, if this centrifugal force is used, the speed of centrifugation must be kept very constant from to time in order to give reproducible results. Also, the duration of centrifugation is critical if it is kept much below 1 hour.

Jackson & Nutt (1951) showed that, increasing centrifugal force *i. e.* up to 5000 g, the amount of trapped plasm comes smaller and that both the speed duration of centrifugation become less c for the result. In recent years, hema centrifuges using capillary tubes and greater centrifugal forces, up to 10,0 have become commercially available an now in use in the majority of laborat The characteristic properties with rega the packed red cell volume of one such trifuge (International Micro-Capillary trifuge, Model MB) has been reported Govern, Jones & Steinberg, 1955). amount of trapped plasma obtained d these conditions was investigated by (1956), who found that the trapped p in the packed cell columns was 2.0 pe with a standard error of a single deter tion of 1.8 per cent. Since both the value and the variation between deter tions in Furth's work seem unexpe large, a re-investigation of the proble undertaken.

## METHODS

lood from five healthy individuals, two females three males was used. It was taken from a al vein into a heparinized syringe and trans- d to 10 ml centrifuge tubes. Each sample was ifuged at about 1000 rpm for 5 minutes and plasma removed and saved. The buffy coat removed and the cells resuspended in their inal plasma. This procedure was repeated , but, before the final resuspension, about ml of a gel filtered radioiodinated human n albumin solution (see below) was added he plasma. The labelled plasma and the red were mixed to give four portions of blood different hematocrit values. From each por- two or three capillary tubes (Capillary Tubes, arinized, Aloe Scientific Division of A. S. Company, St. Louis, Mo., U.S.A.) were filled o about 60-70 mm and spun in the centrifuge below) for exactly 10 minutes. The packed red volume was estimated to the nearest third re and the tubes were cut immediately below packed red cell meniscus. The cut tubes were ed in plastic tubes fitted to a well scintillation tal and counted. Corrections for varying geo- y due to the different cut tube lengths were e using standard solutions. Each sample of cut s was counted to a counting (random) error of than 5 per cent. The percentage of trapped na (per cent TP) in the packed red cell column CC) was calculated with the formula

$$\text{cent TP} = \frac{\text{count rate in PRCC} \times (1 - \text{Htc}) \times 100}{\text{count rate in plasma and PRCC} \times \text{Htc}}$$

*he radioiodinated human serum albumin* was ned from the Radiochemical Centre, Amers- England. It was used about one week after tch and contained about 400 $\mu$c and 20 mg of nin per ml. Less than one hour before use, it subjected to gel filtration through polydextran s ("Sephadex" type G-25, manufactured by macia Ltd., Uppsala, Sweden) according to details described by Bill, Marsden & Ulfen- (1960). The middle portion of the first peak dioactivity was used for the experiments. An ot of this portion was subjected to vacuum tion through a protein-tight membrane ("Kol- m-Hülzen nach Miess", Membranfiltergesell- schaft, Göttingen, Germany) about 20 hours after its use, during which time it was kept at room temperature for 4 hours and at —20° C for 16 hours. 5 ml of the solution was pipetted into a membrane tube. After about 5 minutes of filtration, 2 ml of the solution had been filtered. The concentration of the radioactivity in the filtrate divided by that of the original solution was taken to be the fraction of free iodine in the original solution.

*The centrifuge* (International Micro-Capillary Centrifuge, Model MB) gave 10900 rpm at 220 volts as measured with a stroboscope. This corresponds to between 8770-10500 g in the middle of the PRCC at hematocrits of 40 and 20 per cent respectively (mean effective radium of centrifugation being 66 and 79 mm respectively).

## RESULTS

The ultrafiltrate of the previously gel filtered $I^{131}$-albumin solution contained 0.254 per cent of the total radioactivity concentration.

The amount of trapped plasma, expressed as percentage of the packed red cell column, was 1.31 per cent in a total of 20 determinations. The standard error of a single determination was 0.106 per cent. Analysis of the source of variation (Snedecor, 1957), showed that the error of a single determination due to methodological errors was 0.092 per cent and that the error due to differences between individuals was 0.051 per cent. All data are plotted in Fig. 1 as a function of the hematocrit. The regression of trapped plasma upon hematocrit is per cent TP = —0.00103 Htc + 1.35 and the standard error of the slope is 0.00132.

The difference between the packed red cell column of 10 blood samples (hematocrit range 15.5 — 62.3 per cent) spun at 3 and 10 minutes respectively was 0.35 per cent (standard error of mean 0.06 per cent). The difference between the packed red cell

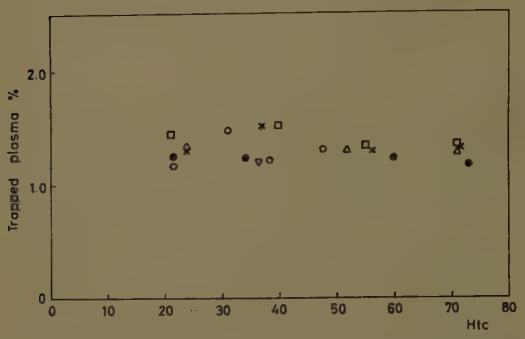


Fig. 1. The amount of trapped plasma, expressed as per cent of the packed red cell volume, in 5 normal individuals at different hematocrit values.

columns of the same blood samples spun at 10 and 20 minutes respectively was 0.02 per cent (standard error of mean 0.05 per cent).

## DISCUSSION

Jackson & Nutt (1951) used Evans Blue Dye for determinations of the trapped plasma and showed that the proportion of trapped plasma decreased as the relative centrifugal force increased. Their results, the lowest value of which was between 1-3 per cent at the highest g-value of between 4000-5000 g, can be extrapolated to the centrifugal force used in the present work, *i. e.* about 10,000 g, to give about 1 per cent which is in good agreement with our results.

Furth (1956) used chromic ($Cr^{51}$) chloride labelled plasma proteins as volume indicator in determinations of trapped plasma. The relative centrifugal force was probably identical to that used here, but the duration of centrifugation was kept at 6 minutes. The mean value in 36 determinations was 2.0 per cent, but the variation between the determinations was very large, corresponding to a standard deviation of 1.8 per cent. The reason for this is not clear, but since there was always some adsorption of chromic i on the red cells it is reasonable to expec larger variation.

The present study shows that the amount trapped plasma obtained after 10 minutes centrifugation with a relative centrifu force of about 10,000 g is not more than per cent and that this can be estimated w great precision. Furthermore, the variati between normal individuals seems to small. The investigation also confirms a extends the observations of Jackson & N (1951) that, under greater centrifugal forc the amount of trapped plasma is independ of the hematocrit value and thus of smal variations in the centrifugal force. The fluence of varying duration of centrifugati on the amount of trapped plasma was studied in the present investigation. Ho ever, from the measurements of the pack red cell column after centrifugation at 3, and 20 minutes respectively, some conc sions can be drawn on this point. The pack red cell column decreased significantly tween 3 and 10 minutes of centrifugation, the difference is wholly due to difference the content of trapped plasma, it may be ferred that the trapped plasma after 3 m utes centrifugation is about 2 per cent. Usi the same arguments, it may be inferred t no further decrease in trapped plasma is tained between 10 and 20 minutes of cent fugation.

The two possible systematic errors pres in this investigation, as well as in all simi studies, namely the possible presence of f iodine (or Evans Blue) in the plasma a the possible adsorption of labelled albu (or Evans Blue) to red cells, will both te to give falsely high values for the trap

ma. The first mentioned possible error certainly very small in this study, since gel filtration was clearly very effective in oving free iodine from the $I^{131}$-albumin tion. The second possible complication arise if there are denatured albumin ecules in the solution used for labelling if these become adsorbed on to the red s. The importance of this possible phenon cannot at present be assessed. At any e, it can be stated with some confidence t the amount of trapped plasma obtained ler the centrifugal conditions used here is more than 1.1—1.5 per cent of the ked red cell column.

## SUMMARY

The amount of trapped plasma present in ked red cell columns after centrifugation 10 minutes at 8770—10500 g in capillary es was investigated. Gel filtered $I^{131}$-umin containing less than 0.25 per cent of free iodine was used as plasma label. The amount of trapped plasma in red cell columns obtained under such conditions was 1.31 per cent and independant of the hematocrit value.

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# RENAL TUBULAR PERMEABILITY TO UREA

## WITH SPECIAL REFERENCE TO ACCUMULATION OF UREA IN THE RENAL MEDULLA.

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In man and dog, 10-60 per cent of the filtered urea is reabsorbed in the tubules. From his studies on urea excretion on different types of diuresis, Shannon (1936, 1938) suggested that the proximal tubules are most permeable to urea, and that the main portion of urea loss occurs from this segment. In the present study the stop-flow technique introduced by Malvin, Wilde & Sullivan (1958) has been used to obtain more direct and detailed information about urea excretion. It was of special interest to study possible back diffusion from the collecting ducts, since this structure has recently been shown to be highly permeable to urea (Klümper, Ullrich & Hilger, 1958, Kiil & Aukland, 1960 b). However, the demonstration of passive net outflux of urea (net flux out from the tubular lumen) depends on a concentration gradient for urea across the tubular wall. This prerequisite may not be fulfilled for the tubular segments situated in the renal papilla, which has a high urea concentration (Ullrich & Jarausch, 1956). It was therefore of inter to study transtubular movement of labell urea. This can be done by *i. v.* injection urea-$C^{14}$ during the last minutes of a ma nitol stop-flow period of 10-15 minu duration. At this time the glomerular fi ration is stopped whilst renal blood flow reduced by less than 50 per cent (Kiil Aukland, 1961 b). Appearance of labell urea in the stop-flow portions will therefo indicate movement of urea from peritubul blood into the lumen through the tubul wall.

Urea back diffusion from the proxim tubules might possibly provide informati about the mechanism of osmotic diuresis. has been assumed that osmotic diuret act mainly by reducing salt and water absorption in the proximal tubules, wh the sodium reabsorption process is thoug to be limited by a relatively small concent tion gradient across the tubular wall (Smi 1951). In mannitol stop-flow experimen sodium concentration of proximal samples nearly unaltered compared to free flow co trol values, probably indicating that the ma imal sodium gradient has been reached

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dy during free flow on high mannitol diu-
s (Aukland & Kiil, 1961), as first sug-
ted by Wesson & Anslow (1948). If this
cept is correct, one would expect that
ximal back diffusion of urea during stop-
v would allow further reabsorption of salt
water. To test this hypothesis, a com-
ison was made between stop-flow experi-
nts with normal and with raised plasma
a concentration.

f urea is lost from the proximal tubules
diffusion, a higher percentage of filtered
a would disappear from the urine if the
v rate in the proximal tubules is reduced.
is was studied by reducing glomerular
ration rate (GFR) by means of increasing
ureteral pressure during osmotic diuresis
iil & Aukland, 1961 b).

As first shown by Shannon (1936), urea
rance is higher during increasing urine
v than at a stable flow of the same magni-
e ("exaltation of urea clearance"), and
y even exceed the glomerular filtration
e. The origin of this "extra" urea excre-
n is not clear, but it has recently been sug-
ted that urea is washed out from the renal
dulla at increasing urine flow (Ullrich &
ausch, 1956, Schmidt-Nielsen, Osaki,
rdaugh & O'Dell, 1958). An attempt was
refore made to find out if the loss of urea
n the medulla is sufficient to account for
extra amount of urea appearing in urine
ing increasing urine flow. A rapid in-
ase of urine flow was obtained by sudden
uction of an increased ureteral pressure.
e extra urea excretion compared to the
retion rate expected at the actual urine
v was calculated and compared to the
ulated loss of urea from the renal
dulla.

## METHODS

All experiments were performed upon mongrel dogs on mixed diet, weighing from 12 to 25 kg. Anesthesia was induced by Nembutal-sodium *i.v.*, 25 mg/kg body weight. Subsequent smaller doses were added to maintain light anesthesia.

The stop-flow experiments were performed during mannitol diuresis according to Malvin *et al.* (1958). A detailed description of the experimental procedure has been given in a previous paper (Aukland, 1960). Ten experiments were carried out at normal plasma urea concentration, 15-50 mg/100 ml. In four experiments plasma urea concentration was raised to 150-600 mg/100 ml and kept at a stable level by means of priming and sustaining intravenous infusion of urea. The results are presented graphically in the same manner as in previous papers (Aukland, 1960, Kiil & Aukland, 1960). Creatinine (Cr) was included to indicate water reabsorption, and sodium and para-amino hippurate (PAH) for identification of the various nephron segments: The distal tubules are indicated by sodium reabsorption and the proximal tubules by PAH secretion. Filtrate formed after reinstatement of flow was indicated by appearance of inulin (or ferrocyanid) injected *i.v.* 45 seconds prior to unclamping the catheter.

In one stop-flow experiment of 15 minutes duration, 1000 $\mu$c of urea-$C^{14}$ was injected *i.v.* together with 0.5 g inulin during the last 2 minutes of the stop-flow period. In order to obtain a relatively stable plasma concentration, the infusion rate was adjusted so that about 2/3 of the labelled urea was given in the first half minute and the last 1/3 during the remaining part of the stop flow period. Filtrate formed after reinstatement of flow was indicated by *i.v.* injection of 10 ml 10 per cent ferrocyanide 45 seconds prior to unclamping. If labelled urea enters the tubules through the glomeruli, its appearance curve in the stop-flow diagram would be expected to coincide with that of inulin. If, on the other hand, urea diffuses from the peritubular circulation into the lumen across the tubular wall, it should appear in more distal samples than inulin. However, a faster diffusion rate of urea along the tubular lumen, due to the widely different molecular weight of urea and inulin, might confound the interpreta-

tion. In another experiment of this kind, urea was therefore injected together with ferrocyanide, whilst new filtrate was indicated by inulin.

The effect of increased ureteral pressure on urea excretion was studied in three experiments during osmotic diuresis (mannitol or urea), and in six experiments during hypertonic salt diuresis induced by continuous infusion of Pitressin (Parke-Davis & Co.) at a rate of 100 mU/min. Ureteral pressures from 30 to 158 cm water were used. A detailed description of the counter pressure experiments is given elsewhere (Kiil & Aukland, 1961 a, b). In four experiments the effect of sudden reduction of ureteral pressure on urea excretion was studied by continuous sampling of small urine portions (1-2 ml) for 2-3 minutes, and thereafter in periods of 2 minutes duration. Two of these experiments were performed during "pitressin diuresis" and the other two during water diuresis.

In all experiments a stable plasma concentration of Cr and PAH was provided by a priming dose of these substances followed by a sustaining infusion by means of an electric infusion pump. The chemical determinations of sodium, potassium, creatinine, PAH, ferrocyanide, inulin and urea were made with the same methods as previously reported (Kiil & Aukland, 1960 a). Urine and plasma osmolarity was calculated as Cr + Urea + 2 (Na + K), measured in mOsm/l. Urea-$C^{14}$ was measured in a Friseke & Hoepfner automatic window gas flow counter. Urine and plasma samples of 0.2 ml were counted for a period of time to give not more then 1 per cent counting error. Since the weights of the samples were low and nearly equal, no correction was made for self-absorption. Specific activity was calculated as counts/sec. mg. In order to compare the appearance curves for urea-$C^{14}$, inulin and ferrocyanide, the concentrations were recorded in percent of the highest value obtained.

## RESULTS

### *Ordinary stop-flow technique.*

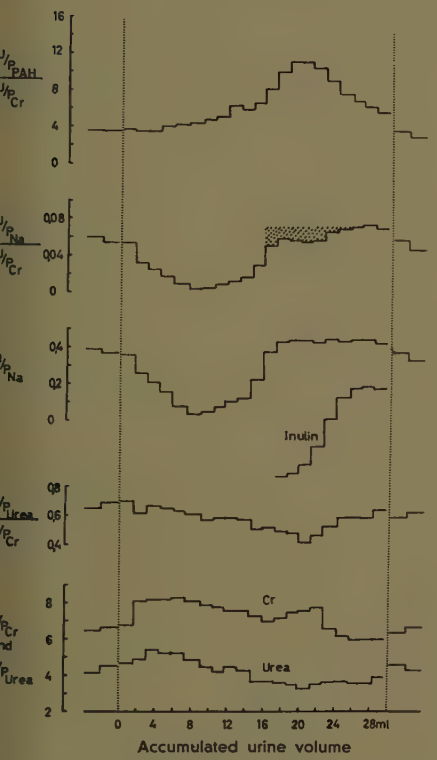
A typical result obtained in ordinary mannitol stop-flow experiments is shown in Fig. 1 a. In the proximal samples urea concentration index ($U/P_{Urea}$) had been lower compared to creatinine concentration index ($U/P_{Cr}$), indicating that urea had diffused appeared from these samples during the stop-flow period. This is even more clearly demonstrated by the lowering of the urea excretion ratio (ratio between excreted and filtered amounts of urea, $U/P_{Urea}/U/P_{Cr}$). In all experiments the lowest urea concentration was found in the proximal samples (Figs. 1 a, b and 2), showing that most urea had been lost from the proximal tubules.

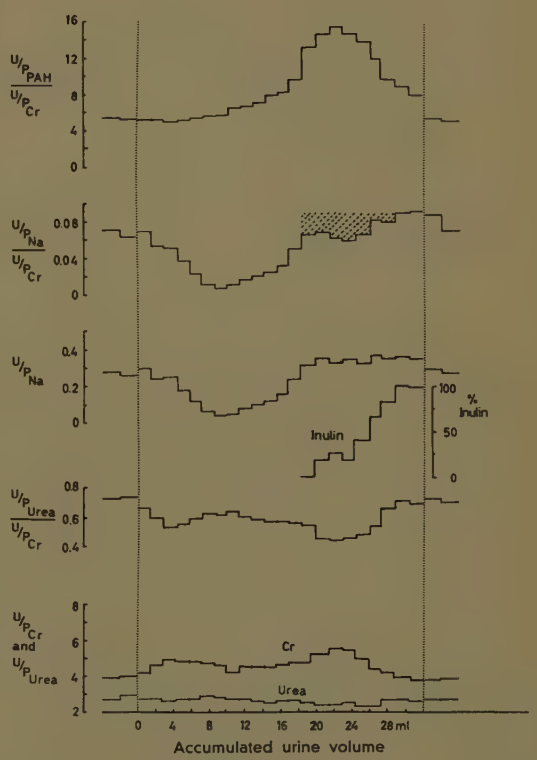
Urine samples arrested in the distal tubules had an urea excretion ratio equal to or slightly lower than free flow controls, indicating that only small amounts of urea have been lost from these samples during stop-flow. In protracted experiments, however, considerable urea loss might occur also from the distal tubules, as shown in Fig. 2, where the ureter was clamped for 14 minutes.

The behavior of urea in the most distal samples, presumably representing the collecting ducts, showed no consistent pattern (Figs. 1 a, b and 2). In most experiments urea concentration increased slightly, generally accompanied by slight net outflux of urea, as indicated by lowering of urea excretion ratio. In other experiments urea concentration was maintained at free flow level with or without net outflux of urea.

No evidence of urea net influx was found in any nephron segments.

The effect of high plasma urea concentration was studied in four experiments. After a control stop-flow, plasma urea concentration was raised to 150-600 mg/100 ml. The sustaining infusion of mannitol, Cr and PAH was maintained unaltered. After approximately 1 hour for equilibration, the stop-f




. 1 a and b. Showing proximal back diffusion of
and its influence on sodium and water re-
orption. Dog weight 22 kg. Control clearance
ods plotted before and after the vertical broken
respectively.

erimental protocol:

8 min: Priming infusion: 1 g Cr, 0.1 g PAH and 30 g mannitol in 150 ml 0.45 % NaCl solution.

-95 min: Sustaining infusion I: 1.5 g Cr, 0.4 g PAH and 150 g mannitol in 1000 ml 0.45 % NaCl. Infusion rate 8 ml/min.

-77 min: Clearance periods 2 min × 2. Urine flow 6.9 ml/ min.

-83 min: Ureteral catheter occluded. Infusion rate reduced to 6 ml/min.

5—82.50 min: 3 ml 5 % inulin *i. v.*

83—87 min: Continuous collection of 25 urine samples. (Fig. 1 a).

87—91 min: Clearance periods 2 min × 2. Urine flow 7.1 ml/min.

95—109 min: 22.5% urea added to sustaining I. Infusion rate 8 ml/min.

109—175 min: 3 % urea added to sustaining I. Infusion rate 8 ml/min.

158—162 min: Clearance periods 2 min × 2. Urine flow 8.6 ml/min.

162—168 min: Catheter occluded. Infusion rate reduced to 6 ml/min.

167.25—167.50 min: 3 ml 5 % inulin *i. v.*

168—171: Collections of 28 samples. (Fig. 1 b).

171—175: Clearance periods 2 min × 2. Urine flow: 8.5 ml/min.

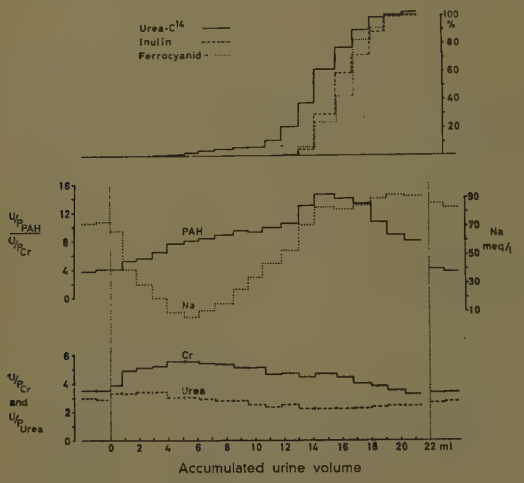



Fig. 2. Showing transtubular movement of urea-$C^{14}$.

Dog weight 15 kg.

Experimental protocol:

0—5 min: Priming infusion: 0.5 g Cr, 0.2 g PAH and 20 g mannitol in 100 ml 0.9 per cent NaCl. 5 min: Start sustaining infusion of 1.5 g Cr, 0.4 g PAH and 150 g mannitol in 1000 ml 0.9 per cent NaCl at a rate of 8 ml/min. 30—34 min: Clearance periods 2 × 2 min. Urine flow 8.7 ml/min. 34 min: Ureter occluded. Infusion rate reduced to 6 ml/min. 46—47.25 min: 800 $\mu$c Urea-$C^{14}$ and 0.25 g inulin in 25 ml saline *i. v.* 47.25—47.50 min: 200 $\mu$c urea-$C^{14}$ and 1 g sodium-ferrocyanide in 10 ml water *i. v.* 48 min: Ureteral catheter unclamped. Continuous collection of 30 urine samples. 50—54 min: Clearance periods 2 × 2 min. Urine flow 9.7 ml/min. Control clearance periods plotted before and after the vertical broken lines respectively.

procedure was repeated. One experiment of this kind is presented in Fig. 1 a and b. Due to the additional diuretic effect of urea, urine flow increased considerably from the first to the second stop-flow period, with a concomitant drop in $U/P_{Cr}$ from approximately 6.5 to 4 in control periods. The curves of urea excretion ratio were very similar, however, apart from a decrease in the most dis samples in the high urea period. (Th difference was not observed in other expe ments.) In consequence of the high urea co centration of glomerular filtrate in the seco series (Fig. 1 b), the proximal urea outfl was about 15 times larger than in the cont period (Fig. 1 a). As had been expecte this allowed a larger proximal reabsorpti of sodium and water. The relatively grea increase of proximal $U/P_{Cr}$ ratios in pro mal urine portions in the high urea peri suggests a larger water reabsorption than the control experiment. However, due replacement of reabsorbed fluid by new fi rate containing creatinine, no accurate calc lation of additional water reabsorption c be made.

Sodium concentration (or $U/P_{Na}$) proximal portions was maintained appro mately at free flow level in both experimen However, net outflux of sodium during t stop-flow period, as indicated by lowering sodium excretion ratio ($U/P_{Na}/U/P_C$ was larger during the high urea experime as indicated in Fig. 1 a and b, by cro hatched areas.

Similar results were obtained in anoth three experiments of this kind.

*Movement of peritubular urea-$C^{14}$ across the tubular wall.*

In two experiments it was shown th labelled urea may pass from peritubu blood into the proximal tubules. As demo strated in Fig. 2, urea-$C^{14}$ appeared in m distal samples and in relatively higher co centration than inulin injected simultaneo ly. In urine portions corresponding to t distal tubules, only traces of labelled u

e found, and in the most distal portions, sumably representing the collecting ducts, ctivity could be detected. The close rela- ship between inulin and ferrocyanide in- ed with an interval of 1½ min, showed the glomerular filtration had been vir- ly stopped.

n another experiment urea-$C^{14}$ was in- ed together with ferrocyanide to mini- e the confounding effect of possible urea usion from glomeruli along the tubular en. Also in this case urea-$C^{14}$ appeared rly ahead of ferrocyanide, indicating that lled urea had gained access to proximal ular urine through the tubular wall. No ct measurement of this transfer is given hese experiments. However, the vertical ance between the curves of urea-$C^{14}$ and in (Fig. 2) gives a rough estimate of a activity due to transtubular transfer. specific activity of proximal samples in both experiments far lower that in ma, showing that equilibration was not ined in the course of 2 and 5 minutes pectively.

### *ect of increased ureteral pressure urea excretion.*

he effect on urea excretion of reducing R by increased ureteral pressure is shown Fig. 3. In five experiments on osmotic resis, ureteral pressures from 50-158 cm er (average 100 cm) were used, and R was reduced by an average of 65 per of free flow control values. In five ex- ments on hypertonic salt diuresis, press- s from 30 to 80 cm (average 50 cm) were d, and GFR was reduced only by an rage of 12 per cent. Periods without nter pressure were also included as con-

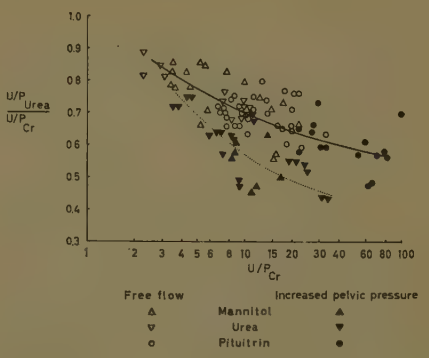



Fig. 3. Effect of increased pelvic pressure on urea excretion during osmotic diuresis and hypertonic salt diuresis. 98 clearance periods in 9 dogs. Broken line drawn through values obtained during increased pressure on osmotic diuresis with marked reduction of GFR (filled triangles). Unbroken line represents all other clearance periods with normal or slightly reduced GFR (open symbols and filled circles).

trols. It was found (Fig. 3) that the urea excretion ratios in osmotic diuresis counter pressure experiments were lower at all $U/P_{Cr}$-ratios, compared to the values obtained during free urine flow or at moderate counter pressure during salt diuresis. This means that a higher percentage of filtered urea is reabsorbed when GFR is reduced.

In Fig. 3 urea excretion ratio has been plotted against creatinine concentration index in the conventional manner (Shannon 1936). When GFR is unaltered, this is equal to plotting against urine flow. If GFR is reduced, however, a given $U/P_{Cr}$ ratio will correspond to a lower urine flow than at normal GFR, as seen from the formula GFR = $U/P_{Cr}$. V (V = urine flow). One might therefore suspect that the increased back diffusion demonstrated above might be due to reduction of distal flow rate and not to reduction of GFR. However, when the values in Fig. 3 are plotted against urine flow (ml/min kg body weight), there is still a marked difference between osmotic diuresis counter pressure and free flow.

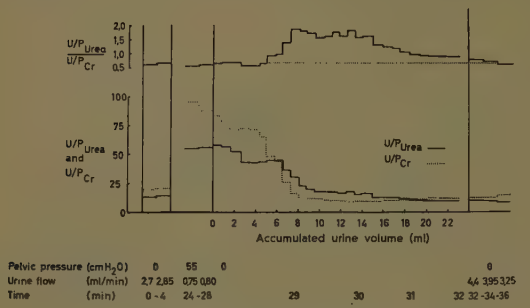



Fig. 4. Washout of medullary urea by sudden increase of urine flow produced by reduction of pelvic pressure. Dog weight 20 kg. Sustaining infusion: 2 g Cr, 0.3 g PAH and 10 U Pitressin in 1000 ml 0.45 per cent NaCl, at a rate of 8 ml/min. Other data given in figure and in Table I, columne 1.

### *Urea excretion after sudden reduction of ureteral pressure.*

The effect of a rapid increase of urine flow was studied by sudden reduction of ureteral pressure from 55-80 cm water to zero. Fig. 4 shows an experiment performed during hypertonic salt diuresis (data given in Table 1, experiment No. 1). Concomitantly with reduction of creatinine and urea concentration, the urea excretion ratio increased steeply to 1.88 and then declined gradually and reached normal level 6-7 minutes after reduction of ureteral pressure. It was thus clearly demonstrated that the amount of urinary urea may greatly exceed the filtered amount for a short period of time. A similar outflow pattern was observed in two experiments during water diuresis. The increase of urea excretion ratio was more protracted, however, and did not attain so high values as during antidiuresis.

In the following an attempt is made to estimate whether urea loss from the medulla can account for the excessive urea excretion following reduction of ureteral pressure.

The excess of urea appearing in urine was ca lated separately for each urine portion as the t amount of urea minus the amount expected if u excretion had paralleled creatinine excretion:

$$M_n = (U/P_{Urea_n} - U/P_{Cr_n} \cdot k)\ P_{Urea}\ V_n$$

$M_n$ is the excess of urea in portion n, $U/P_{U}$ and $U/P_{Cr_n}$ the concentration index for urea creatinine in this urine portion, $P_{Urea}$ = pla urea concentration and $V_n$ = volume of sample The factor k is the urea excretion ratio corresp ing to the $U/P_{Cr}$ ratio reached after reductio ureteral pressure. In the experiment shown Fig. 4, a value of 0.70 was used for k, based observations at corresponding $U/P_{Cr}$ ratios du free flow periods in the same experiment. By ad the excess of urea from all urine portions, a t excess of 29.8 mg urea was calculated in this periment.

The calculation of the simultaneous loss of u from the renal medulla is based on the follow presumptions:

1. Urea concentration rises linearly from cortico-medullary border to the tip of the pa (Ullrich & Jarausch, 1956).

2. Urea concentration at the tip of the papill equal to urinary urea concentration (Ullrich Jarausch, 1956).

3. Urea concentration at the cortico-medul border varies from 2-6 times plasma urea con tration, dependent on urine flow (Own unpubli observations, Ullrich & Jarausch, 1956). In particular experiment this value had to be estim rather approximately. Because this value is s compared to the concentration at the tip of papilla, no large error will result from approximation.

4. The medulla accounts for 29 per cent of total kidney volume in the dog (Ullrich, 19 Specific weight is taken as 1.

5. Medullary urea space is estimated to 80 cent of total medullary volume.

To simplify the calculation, the renal medul first considered as a quadrangular pyramid side s and height H. Urea concentration at

called $C_1$ and at the base $C_2$. From Fig. 5 rived:

ie side at a distance h from the tip:

$$s_h = \frac{s\,h}{H}$$

rea concentration at the same level:

$$C_h = C_2 + (C_1 - C_2)\,\frac{H-h}{H}$$

ie urea content of a polyhedron with side $s_h$, t dh and concentration $C_h$:

$$dM = s_h{}^2 C_h\,dh$$

substituting equation 1 and 2 in equation 3:

$$dM = \frac{s^2}{H^3}\,(h^2H\ C_1 + h^3\,C_2 - h^3\ C_1)\,dh$$

ie total urea content of the pyramid is then ned by integrating h from 0 to H:

$$= \frac{s^2\,H}{12}\,(C_1 + 3C_2) = \tfrac{1}{3}\ s^2H\ \tfrac{1}{4}\ (C_1 + 3C_2)$$

$\tfrac{1}{3}\ s^2\ H$ is the volume (V) of the pyramid, $C_1 + 3C_2)$ must be the mean urea concentra- The formula may therefore be written:

$$M = V\ \tfrac{1}{4}\ (C_1 + 3\,C_2)$$

is formula is applicable to all pyramids, ir- ctive of s and H. If the renal medulla is con- d as a sector of a sphere, it may be imagined nsist of innumerable small pyramids with the at the corticomedullary border and the tips e tip of the papilla. Also an ellipsoid, or a r thereof, may be composed of pyramids with al volum equal to that of the ellipsoid. The la should therefore also be valid for the lla of a single papilla kidney.

ie calculation of medullary urea content in the iment shown in Fig. 4 is given as an example, at 55 cm ureteral pressure ($M_1$) and then equilibration at zero pressure ($M_2$). Data are in Table I, experiment No. 1.

edullary urea space:

$$60 \cdot 0.29 \cdot 0.80 = 14\ \text{ml}$$

dullary urea contents:

$$14 \cdot \tfrac{1}{4}\ (10.03 + 3 \cdot 1.0) = 45.6\ \text{mg}$$

$$14 \cdot \tfrac{1}{4}\ (1.75 + 3 \cdot 0.5) = 11.4\ \text{mg}$$

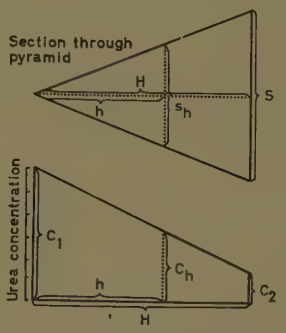



Fig. 5. Schematic model for calculation of medullary urea content. See text for explanation.

Urea loss from the medulla is therefore

$$M_3 = M_1 - M_2 = 45.6 - 11.4 = 34.2\ \text{mg}.$$

Levinsky & Berliner (1959) found that the urea concentration at the tip of the papilla was approximately 20 per cent lower than urinary urea concentration. This would also be expected from the finding of a considerably lower urea concentration within the loops of Henle than in the collecting ducts at corresponding level (Gottschalk, Lassiter, Mylle, Ullrich, Schmidt-Nielsen & O'Dell, 1960). The medullary urea content might therefore be lower then calculated above. On the other hand, the formula evolved probably gives too low an estimation of mean concentration for the following reason: The renal papilla in dogs is of the crest type rather than of the single papilla type, and can therefore not be composed of pyramids, which inevitably give a single tip. If, however, the calculation of mean concentration is based on a prism, giving the formula $M = V\tfrac{1}{3}\ (C_1 + 2C_2)$, obviously too high mean concentration is reached. The correct mean concentration will probably be somewhere between these two formulas.

In the experiment described above, the calculated loss of urea from the medulla was sufficient to account for the excess of urea appearing in urine. As shown in Table I, this was the case in all four experiments, two of which were performed during water diuresis and two during antidiuresis.

Table I. *Extra amount of urea excreted following sudden increase of urine flow co pared to calculated loss of urea from the renal medulla.*

| Experiment No. | 1 | | 2 | | 3 | | 4 | |
|---|---|---|---|---|---|---|---|---|
| Ureteral pressure, cm $H_2O$ | 65 | 0 | 80 | 0 | 55 | 0 | + | |
| $U/P_{Cr}$ | 90 | 14 | 53 | 11.1 | 45 | 16 | 22 | |
| $U/P_{Osm}$ | 2.87 | 1.26 | 2.51 | 1.27 | 0.60 | 0.29 | 0.79 | |
| $U_{Urea}$ (=$C_1$), mg/100 ml | 1003 | 175 | 916 | 220 | 594 | 232 | 585 | 16 |
| $C_2$ (estimated) mg/100 ml | 100 | 50 | 100 | 60 | 90 | 60 | 150 | 12 |
| $P_{Urea}$, mg/100 ml | 17.8 | | 28.6 | | 22.2 | | 45.4 | |
| Kidney weight, g | 60 | | 60 | | 66 | | 36 | |
| Excess urinary urea, mg | 29.8 | | 23.6 | | 10.7 | | 4.8 | |
| Urea loss from medulla, mg | 34.2 | | 28.6 | | 18.2 | | 10.5 | |

## DISCUSSION

Tubular secretion of urea has been demonstrated in amphibians and in certain rodents (for references see Schmidt-Nielsen, 1958). In dog and man, however, no proof of active tubular reabsorption or secretion has been produced. On the other hand, Schmidt-Nielsen and coworkers have recently postulated a limited active transport of urea by the thick ascending portion of the loops of Henle also in sheep, dog and man, in order to explain the dependency of intrarenal urea distribution and urea clearance on protein or urea feeding in these species (Murdaugh, Schmidt-Nielsen, Doyle & O'Dell, 1958, Schmidt-Nielsen, Murdaugh, Osaki & O'Dell, 1957, Schmidt-Nielsen & O'Dell, 1959). In the present study no evidence for active handling of urea was found. It seems reasonable therefore to discuss the findings in terms of glomerular filtration and restricted back diffusion of urea.

*Proximal loss of urea.*

Based on the finding that urea/inulin clearance ratio increases rapidly towards unity during increasing osmotic diuresis, Shannon (1938) suggested that the proxi tubules in dog and man are relatively meable to urea. The reduced urea loss du high osmotic diuresis was attributed to rate of urine flow in the proximal tub and a low urea concentration of tubular f under these circumstances. Lassiter, schalk & Mylle (1960), by micropunct studies in nondiuretic rats, found that al half of the filtered urea was lost in the pr mal tubules. The present study supp these findings by demonstration of ma urea loss from proximal stop-flow sam and furthermore by demonstration of m ment of peritubular labelled urea across walls of the proximal tubules. The co of proximal urea loss by simple diffu also explains the relatively greater urea when GFR was reduced by increased teral pressure. The same conclusion arrived at by Shannon (1936, 1938) Mudge, Foulks & Gilman (1949). Effe (1951 a, b) on the other hand, found in bits that back diffusion of urea was pendent of variations in GFR between and 5 ml/min. It can not be excluded, ever, that these large variations in

e due to variation in the number of active
neruli and not to varying filtration rate
ne individual nephrons.

accordance with expection it was de-
strated that an increase of absolute urea
from the proximal tubules during man-
stop-flow was accompanied by an in-
se in proximal sodium and water reab-
tion (Fig. 1 a and b). The following
hanism is suggested to explain this
ing:

roximal sodium reabsorption is limited
a relatively low sodium concentration
lient across the tubular wall. During high
nitol diuresis the maximal gradient is
hed already during free urine flow,
use mannitol keeps water back in the
en whilst sodium is reabsorbed. Further
uction of sodium concentration can there-
not be obtained during stopped flow.
vever, the isosmotic sodium reabsorption
ng free flow has increased the concen-
on of urea and other poorly reabsorbable
tes in the proximal tubules. When given
le time, urea diffuses out, presumably
isosmotic amounts of water. Outflux
vater in turn tends to raise intraluminal
um concentration and reduce the existing
entration gradient, thereby allowing
ner reabsorption of sodium with main-
nce of proximal sodium concentration
transtubular sodium gradient. Increasing
concentration of the glomerular filtrate
eases the absolute urea loss from the
imal tubules, thereby permitting larger
unts of sodium and water to be reab-
ed.

*al loss of urea.*

he distal convoluted tubules situated in the cortex are probably surrounded by interstitial fluid having a urea concentration nearly identical to that of plasma. The requirement for passive back diffusion is therefore present in this segment. The conditions are more complex when dealing with the loops of Henle and the collecting ducts situated in the medulla, because urea concentration of tissue slices increases towards the tip of the papilla, where it equals that of urine (Ullrich & Jarausch 1956). Klümper *et al.* (1958) showed by microcatherization studies in hamster that urea diffuses out from the collecting ducts within the papilla. They suggested that urea is trapped in the papilla due to the counter current flow in the vasa recta (Wirz 1953), whilst water is removed, thereby creating a high interstitial urea concentration. Evidence supporting this concept was recently obtained by demonstrating a marked loss of urea from the most distal urine portions in nonosmotic stop flow experiments, in antidiuresis as well as in water diuresis (Kiil & Aukland, 1960 b). The basis for urea loss from the collecting ducts in these experiments was suggested to be the continued sodium and water reabsorption from the distal tubules, raising the urea concentration of fluid delivered to the collecting ducts to higher levels than that prevailing in the papilla. It was also shown that the direction of urea flux was reversed when urea concentration within the lumen was rapidly lowered. In the present stop-flow experiments performed during mannitol diuresis, the concentration of urea in the most distal portions remained almost unaltered, and the excretion ratio indicated slight or no urea loss. (The considerable loss of urea from the collecting ducts observed in the experiment

shown in Fig. 1 b may be due to incomplete equilibration of papillary urea at the new, high plasma urea concentration.) As high urea permeability in the collecting ducts must be expected also under these conditions, it must be concluded that the urea concentration of urine and papillary interstitial water is not very different. It also indicates a very high efficiency of the urea trapping mechanism of the medullary counter current system, since papillary urea concentration apparently was maintained at approximately the same level for several minutes, without addition of urea from the collecting ducts.

Labelled urea, injected *i. v.* during stop-flow, failed completely to appear in the most distal urine portions. This is probably not due to low urea permeability in the collecting ducts, but again indicates the high efficiency of the counter current exchange system when urea is concerned. It is assumed that while net flux of urea takes place from ascending to descending limbs of vasa recta, diffusion of urea-$C^{14}$ in the opposite direction may proceed so rapidly that almost no isotope reaches the papilla during the experiment. It can not be excluded, however, that medullary blood flow may be greatly reduced during mannitol stop-flow experiments, thus excluding labelled urea from the papilla.

Although limited, a certain amount of urea must be removed from the papilla *via* the vasa recta, dependent on the flow rate in these vessels. At a given blood flow, urea loss should be proportional to the urea concentration at the tip of the papilla, — in other words, inverse to urine flow. The rate of urine flow will also determine urea back diffusion from the distal tubules. Studies on urea clearance at varying urine flow therefore can not be used to distinguish betwe urea loss from the collecting ducts and distal tubules. Urea may also be remov from the papilla *via* the loops of Henle, suggested by microcatheterization studies rats which showed a higher $U/P_{Urea}/$ $P_{Inulin}$ ratio in early distal samples than proximal samples (Lassiter *et al.*, 1960).

The mechanism for urea concentration the papilla, as described above, depends a low permeability to urea in the distal c voluted tubules, permitting urea to be c centrated by reabsorption of sodium water in this segment. In most stop flow periments only very small amounts of u were lost from distal tubular samples. prolonged experiments, however, the exc tion ratio was also lowered considerably these portions, showing that the distal tu les are not completely impermeable to u (Fig. 2). Micropunction studies in rats ha shown that back diffusion also occurs in t segment (Lassiter *et al.*, 1960). As previo ly pointed out (Kiil & Aukland, 1960 b), proximal portions of the collecting du must also have a low permeability to u allowing mainly reabsorption of water. this were not the case, urea concentrati would not increase towards the papilla.

The mechanism outlined above can account for all observations on urea dist bution within the kidney. Schmidt-Niels & O'Dell (1959) found in proteindeple sheep that the tissue/plasma urea concent tion ratio in the outer zone of the medu might attain as high a value as 115, w the ratio at the tip of the papilla was lo than 20. Urine formed at the same time an even lower urea concentration. authors attributed this peculiar pattern

e urea reabsorption by the ascending ion of the loops of Henle. It is believed, ever, that the observations may be ex- ied without implying active transport of : Obviously, back diffusion of urea from distal portions of the collecting ducts can account for the high urea concentration ne outer medulla. Furthermore, the low concentration at the tip of the papilla ld even demand that urea is removed n the inner medulla. This cannot be ac- plished by the vasa recta, which only s to maintain preexisting concentration lients. However, if protein depletion in- ses urea permeability in the distal part ne nephron (including the ascending limb Henle's loops, the distal convoluted tubu- and the proximal portion of the collecting s traversing the outer medulla), the fol- ng situation would be expected: Urea is centrated in the distal tubules by removal alt and water, diffuses freely out from entire length of the collecting ducts into ascending limb of Henle's loops. Thus ounter current exchange mechanism is blished between the collecting ducts and nding limbs, producing a decrease of concentration within the collecting s and the medulla from the outer zone he tip of the papilla. This mechanism implies a recirculation of urea *via* asc- ng limb-distal tubule-collecting duct. s would tend to raise urea concentration ne distal tubules and increase urea back sion from this segment, in accordance the low urea excretion in protein de- d animals.

he vasa recta would not interfer with the osed mechanism, but it should be noted the descending limb of Henle's loop must be less permeable to urea than the ascending limb.

The finding of a lower urea concentration in urine than in the papilla in protein depleted sheep (Schmidt-Nielsen & O'Dell 1959) might indicate urea diffusion from the papilla into the collecting ducts. However, if this were the case, urea could not be concentrated in the outer medulla by diffusion from the collecting ducts where urea concentration had to be even lower than in urine, and it would seem inevitable to invoke active transport of urea. It seems more reasonable that the lower urinary urea concentration is due to urea loss from the urinary tract, as proposed by Levinsky & Berliner (1959).

Also protein depleted man and dog have a very low urea excretion at low urine flow (Murdaugh, Schmidt-Nielsen, Doyle & O'Dell, 1958, Schmidt-Nielsen, Murdaugh, Osaki & O'Dell, 1957). Analyses of tissue urea concentration in protein depleted dogs have been reported by Levinsky, Davidson & Berliner (1959) and Levinsky & Berliner (1959). They found almost unaltered urea concentration from the inner medually zone to the tip of the papilla, and an equal or slightly lower urinery urea concentration. The mechanism outlined above might also explain these observation, although the increase of urea permeability in the distal part of the nephron would seem to be less striking in protein depleted dogs than in sheep, allowing only restricted back diffusion of urea from the proximal portion of the collecting ducts.

*Exaltation of urea clearance.*

The concept of the exaltation of urea clearance as due to washout of urea from the renal medulla was supported by the present experiments, as it was found that the esti-

mated loss of urea from the medulla was sufficient to account for the excess of urea appearing in urine (Table I). Similar results are obtained if the same calculations are applied to the experiments of Shannon (1936). (Exp. No. 78 B, dog E. Kidney weight estimated from GFR: 84 g. Estimated loss from medulla from period 2 to 4: 65 mg. Excess urinary urea in periods 2 and 3: 32 mg.)

It cannot be decided from the present experiments whether urea is washed out via the loops of Henle and distal tubules, or directly into the collecting ducts. Previous stop-flow studies on nonosmotic diuresis suggested, however, that papillary urea is mainly lost directly from the papilla through the walls of the collecting ducts (Kiil & Aukland, 1960 b).

*Effect of antidiuretic hormone on tubular permeability to urea.*

Jaenike (1961) showed recently that pituitary antidiuretic hormone facilitates the accumulation of urea in the renal papilla in dogs, and suggested that this resulted from an increased permeability to urea in the collecting ducts. The present experiments support this concept as it was observed that the washout of urea was faster on pitressin induced diuresis than on water diuresis. Whether urea permeability in other nephron segments is influenced by antidiuretic hormone or not, cannot be decided.

## SUMMARY AND CONCLUSIONS

Urea excretion was studied in dogs during elevated ureteral pressure and by stop-flow experiments, and transtubular urea diffusion by *i. v.* infusion of labelled urea during flow. The effect of rapid increase of u flow produced by sudden reduction elevated ureteral pressure was studied four experiments.

No evidence for active tubular trans of urea was obtained. In dogs on mixed the distal tubules are highly impermeabl urea, while considerable back diffusion urea occurs from the proximal tubu A high rate of proximal back diffusion ing mannitol stop-flow, induced by eleva of plasma urea concentration, increa proximal sodium and water reabsorpt indicating that a limiting transtubular centration gradient for sodium is obtai already during free flow on high osm diuresis. Transtubular movement of per bular labelled urea could be demonstra only in the proximal tubules.

Reduction of GFR, by elevation of uret pressure during osmotic diuresis, increa the percentual back diffusion of filtered u probably by reduction of proximal flow r

The distal parts of the collecting ducts highly permeable to urea and permit alm free diffusion in either direction. Due to counter current arrangement of the me lary circulation, only small amounts of u are lost from the collecting ducts.

A formula for calculation of medul urea content was evolved. Sudden incr of urine flow led to excretion of an e amount of urea roughly corresponding to calculated reduction of medullary urea tent. The difference between the wash patterns obtained during water diuresis antidiuresis suggested that pitressin creases urea permeability in the collec ducts.

ıe effect of protein depletion on urea ex-
on and distribution within the kidney is
ıssed.

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# THE RENAL EXCRETION OF THIAMINE DURING ADMINISTRATION OF PROBENECID (P-(DI-N-PROPYLSULFAMYL)-BENZOIC ACID

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n a previous study of the renal excretion thiamine in the dog (Haugen 1961) the dings indicated that thiamine is a sub- nce which both at low and at high plasma centrations, is filtered through the glome- , reabsorbed from the tubular lumen and secreted by the tubular cells into the ne. At high concentrations the tubular retory mechanism is preponderant. At levels, however, this secretory mechan- may be difficult to demonstrate because tubular handling of thiamine results in et tubular reabsorption. A way of attack- the problem would be the blocking of the ular transport mechanism by some drug. s paper presents the results obtained the administration of probenecid (p-(di- ropylsulfamyl)-benzoic acid) as a block- agent.

## PROCEDURE AND METHODS

49 year-old healthy male, H. N., was studied er identical conditions regarding diet and activ- out under different experimental conditions re- ing thiamine administration. He was given a lar breakfast every morning conforming with his habits: 200 grams of bread, with butter, and herring, meat, cheese, and vegetables, 2 cups of coffee with cream, 1 glass (150 ml) of milk. Care was taken to keep this diet constant during the experimental periods. The lunch and evening meals were also kept identical from day to day whereas the composition of the dinner meal was allowed to change more liberally. However, no food particularly rich in vitamin $B_1$ was taken.

In three periods consisting of five, three, and two consecutive days, no additional thiamine was given. In another two periods 1 and 5 mg of thiamine hydrochloride were given during the breakfast meal.

In periods identical with these control periods, one gram of probenecid was given during the breakfast meal whereupon another half a gram with 100 ml of water was given one hour after the end of the meal. It was hoped that this schedule of giving the drug would result in a high renal level of probenecid during the experimental periods.

In two healthy individuals, P.H. and I.Ø., thiamine excretion after breakfast was studied under conditions identical with those outlined above. The study was, however, limited to two control days and two probenecid days.

Urine was collected for four hours starting from breakfast. Care was taken to keep this time interval constant from day to day.

A short-time study in the post-absorptive state

Table I. *Thiamine excretion in individual H.N. under different experimental conditions.*

| | Control periods (μg per minute) | Probenecid periods (μg per minute) | Per cent reduction |
|---|---|---|---|
| **A. Dietary thiamine only.** | | | |
| Study No. 1 | | | |
| Day 1 | 0.138 | 0.052 | |
| » 2 | 0.185 | 0.054 | |
| » 3 | 0.145 | 0.076 | |
| » 4 | 0.140 | 0.079 | |
| » 5 | 0.142 | 0.076 | |
| Mean | 0.150 | 0.067 | 56 |
| Study No. 2 | | | |
| Day 1 | 0.141 | 0.058 | |
| » 2 | 0.221 | 0.056 | |
| » 3 | 0.148 | 0.076 | |
| Mean | 0.170 | 0.063 | 60 |
| Study No. 3 | | | |
| Day 1 | 0.102 | 0.030 | |
| » 2 | 0.094 | 0.049 | |
| Mean | 0.098 | 0.040 | 59 |
| **B. In the postabsorptive state.** | | | |
| Period 1 | 0.078 | 0.065 | |
| » 2 | 0.075 | 0.042 | |
| Mean | 0.077 | 0.054 | 30 |
| **C. During administration of thiamine hydrochloride** | | | |
| | (μg per four hours) | (μg per four hours) | |
| Study No. 1 1 mg daily | | | |
| Day 1 | 133 | 111 | |
| » 2 | 118 | 111 | |
| » 3 | 159 | 115 | |
| » 4 | 168 | 120 | |
| » 5 | 174 | 110 | |
| Mean | 150 | 113 | 24.7 |
| Study No. 2 | | | |
| Day 1 | 528 | 579 | |
| » 2 | 430 | 420 | |
| » 3 | 606 | 418 | |
| » 4 | 648 | 412 | |
| » 5 | 565 | 597 | |
| Mean | 555 | 485 | 12.6 |

was also performed. After two control periods about twenty minutes' duration, one gram of p benecid was given orally to individual H. N. A half an hour another two urine samples w collected.

Urine was analyzed for thiamine by the th chrome method (Haugen 1960). Creatinine determined by the alkaline picrate method (Haug 1953).

## RESULTS

Table I shows the results in individ H.N. During the control periods, w dietary thiamine only, the thiamine excreti was on an average 0.150, 0.170, and 0.0 μg per minute. In the corresponding p benecid periods the excretion decreased less than half the control values, the avera excretion being 0.067, 0.063, and 0.040 per minute, respectively.

After administration of 1 mg of thiami hydrochloride orally, an average of 150 were excreted during the following f hours. After probenecid administration th mine excretion decreased by almost 25 p cent, average 113 μg. When the higher d of 5 mg of thiamine hydrochloride was giv probenecid administration resulted in a sm ler reduction of thiamine excretion, 12.6 p cent on the average. However, because overlapping between the control values a the values obtained during probenecid a ministration, the difference is not statistica significant.

In the postabsorptive study, individ H.N. showed a 30 per cent reduction thiamine excretion, from 0.077 to 0.054 per minute.

In individual P.H. the thiamine excret during probenecid administration decrea

ble II. *Thiamine excretion in indivi-s P.H. and I.Ø. during control days and probenecid days.*

| | Control days (μg per minute) | Probenecid days (μg per minute) | Per cent reduc-tion |
|---|---|---|---|
| vidual P.H. | | | |
| ay 1 | 0.088 | 0.050 | |
| 2 | 0.060 | 0.043 | |
| Mean | 0.074 | 0.047 | 37 |
| vidual I. Ø. | | | |
| ay 1 | 0.092 | 0.037 | |
| 2 | 0.100 | 0.060 | |
| Mean | 0.096 | 0.049 | 49 |

n 0.074 to 0.047 μg per minute, a reduc- of 37 per cent. In individual I.Ø. a ewhat higher reduction — 49 per cent — found (Table II).

ndividuals H.N. and P.H. showed un-nged endogenous "creatinine" clearance es during probenecid administration, reas slightly reduced values were found he individual I.Ø. (Table III).

## DISCUSSION

robenecid (and carinamide) are known nhibit the tubular excretion of various tances; the best known are penicillin, -aminohippurate, diodrast, and phenol Probenecid is also very effective as an osuric agent. No effect upon the tubular sorption of glucose or upon the back sion of urea has been observed, nor has effect upon the excretion of creatinine demonstrated. No inhibitory effect n the absorptive processes in the gut is vn.

he administration of probenecid during periods with dietary thiamine intake only resulted in a more than 50 per cent reduction of the urinary excretion of thiamine in individual H.N. and a somewhat smaller reduction in individuals P.H. and I.Ø. The same pattern was obtained after thiamine administration although the percentage reduction was less. Also in the postabsorptive state was there a decrease in thiamine excretion during probenecid administration.

This lowered excretion of thiamine during probenecid administration may be explained (1) by lowered glomerular filtration of thiamine (2) by unchanged filtration but increased reabsorption in the tubules (3) by unchanged filtration and reabsorption but lowered tubular excretion (4) by a combination of two or of all these possibilities.

(1) may be excluded because the administration of probenecid did not to any significant degree interfere with the glomerular filtration rate. Although endogenous "creatinine" clearance is not exactly equal to the

Table III. *Endogenous "creatinine" clearance during control periods and during probenecid periods.*

| Individual | Control periods (ml/minute) | Probenecid periods (ml/minute |
|---|---|---|
| H. N. | 111 | 109 |
| | 96 | 106 |
| | 104 | 97 |
| Mean | 104 | 104 |
| P. H. | 90 | 93 |
| | 122 | 115 |
| Mean | 106 | 104 |
| I. Ø. | 114 | 100 |
| | 118 | 112 |
| Mean | 116 | 106 |

glomerular filtration rate as determined by the aid of inulin, it may serve as a measure of this function, and it may be concluded that the amounts of thiamine filtered through the glomeruli in the control periods and in the probenecid periods were identical.

(This statement is true provided no change of the physical properties of thiamine or plasma proteins occurred during the administration of probenecid. If thiamine were in some way bound to plasma proteins, probenecid might increase the binding and thus give reduced glomerular filtration of thiamine. However, according to Malnic, Silva, Angelis & Gomes (1960) no protein binding of thiamine occurs. This possibility is, therefore, unlikely.)

(2), enhanced tubular reabsorption of thiamine during the administration of probenecid is also unlikely. No such effect upon any substance is known, the main effect of this drug being either depression of tubular secretion (*e. g.* penicillin, diodrast, phenol red, para-aminohippurate) or of tubular reabsorption (urate).

Therefore, the third possibility is the most likely, *viz.* an inhibition of thiamine excretion through the tubular cells by probenecid. Since previous studies, as mentioned above, showed as most likely both glomerular filtration, tubular reabsorption and tubular secretion of thiamine, the present study gives further support to the supposition of a two-way mechanism for the tubular handling of thiamine, not only when large doses are eliminated, but also when the small amounts present in the food are excreted.

The study gives no information as to the site of tubular reabsorption and secretion of thiamine, nor has any knowledge of the transport mechanism been obtained. At h levels of thiamine, studies using the st flow technique might give an idea of the s of tubular secretion. At low plasma lev however, current methods for thiamine det mination are probably insufficient for ex analysis of the volumes of urine obtai during the stop-flow technique. Studies us radioactive thiamine might, however, g valuable information.

## SUMMARY

The renal excretion of thiamine w studied in 3 healthy individuals before a after administration of probenecid (p-(di propylsulfamyl)-benzoic acid. On a regu diet the administration of 1.5 grams of p benecid resulted in a 30 to 60 per cent duction of thiamine excretion. An identi result was obtained when thiamine hyd chloride was given orally. The findings explained by assuming a two-way mechani for the tubular handling of thiamine, re sorption *and* secretion, operating not o when large doses of thiamine are adm stered but also when small amounts are creted.

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# PHYSICAL CHEMICAL PROPERTIES OF URINARY PROTEIN IN A CASE OF MULTIPLE MYELOMA

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he physical chemical properties reported the classical thermal soluble Bence-es protein vary from case to case. Values he range of 2.44—4.40 S for the sedimen-on coefficient ($S°_{20,w}$), 4.7—9.8 $\times 10^{-7}$ /sec. for the diffusion coefficient ($D°_{20,w}$), 00—90,000 for the molecular weight, 4.6—6.7 for the isoelectric point e been published (Rundles, Cooper & lett, 1951, Putnam & Stelos, 1953). The ne of patients with multiple myeloma may tain a protein which has the same electro-retic mobility as Bence-Jones protein, but s to redissolve on boiling. Molecular ghts of 31,000—45,000 for this kind of tein were calculated by Riva, Dialer & ssig (1951) and Deutsch (1955) who re-led it as Bence-Jones protein. This paper orts a study of some physical chemical perties of proteins in the urine and serum 62 years old male patient with multiple eloma.

## METHODS

lectrophoresis of urine and serum was done on id cellulose acetate membrane filter paper. An te-diethylbarbiturate buffer of Michaelis (pH 8.6; ionic strength 0.125) was used. The same buffer at pH 8.2, 7.0 and 5.0, ionic strength 0.1, was used in the determination of the isoelectric point with paper electrophoresis. Electro-osmosis was checked with urea.

Prior to sedimentation and diffusion studies the samples were dialysed against 0.2-M NaCl in 0.01-M phosphate buffer pH 7.3.

The sedimentation behaviour of urine and serum was examined in the Spinco model E analytical ultracentrifuge at 59,780 r.p.m. at 20.0° using a wedge cell for simultaneous run of two protein concentrations (serum: 1.0 and 1.5 per cent; urine: 0.5 and 1.0 per cent).

The diffusion experiments on two urinary protein concentrations (0.4 and 1.0 per cent) were conducted in the microelectrophoresis apparatus of Antweiler at 18.9° C using a diffusion cell and recorded with a built-in interferometric optical system. For calculation of diffusion coefficients the maximum height-area method was applied.

The molecular weights were derived from Svedberg's equation (Svedberg & Pedersen, 1940). A value of 0.75 for the partial specific volume was used.

## RESULTS

The urine of the patient had a protein concentration of 0.4—1.2 per cent. The daily output of protein was 3.2—12.6 g. On heat-

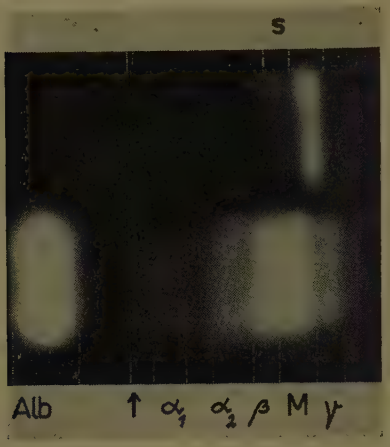


Fig. 1. Electrophoretic patterns of the urine and serum proteins run simultaneously on the same paper. The arrow points to the start. S indicates the smaller fraction of urine protein. Relative concentrations of the serum fractions: alb. 43.7, $\alpha_1$-glob. 1.5, $\alpha_2$-glob. 8.3, $\beta$-glob. 14.0, M-glob. 27.3, and $\gamma$-glob. 5.2 per cent.

ing the urine to 50° C, a precipitate was formed which did not redissolve at 100° C in spite of changing the pH. The serum protein concentration was 7.0 per cent.

The electrophoretic pattern of the serum showed an abnormal fraction M (Fig. 1), which could not be found in a normal serum by the methods available. It amounted to more than one quarter of the total serum protein. It was clearly periodic acid-Schiff-positive and faintly Sudan Black-positive.

On electrophoresis, the proteins of the urine could be separated into two fractions (Fig. 1): a slowly moving main fraction (95.0 per cent) having a mobility intermediate to those of the M- and $\gamma$-fractions of serum and pI 5.6, and a slightly faster moving minor fraction (5.0 per cent). The main fraction was clearly periodic acid-Schiff-positive and Sudan Black-negativ When mixed with the serum, the urina protein moved in the fraction M with no le in sharpness of the latter as compared wi patterns of pure serum.

In the ultracentrifuge, the urinary prote sedimented as a single symmetrical gradie with $d(1/S)/dc$ $0.0255 \times 10^{-13}$ and $S°_{20,}$ 3.93 S (Fig. 2). The diffusion curv showed no skewness and a value of 6.80 $10^{-7}$ cm²/sec. for $D°_{20,w}$ was obtaine From these a molecular weight of 57,7 was calculated. The sedimentation pattern the serum showed an abnormal macroglob lin fraction (Fig. 3).

## DISCUSSION

The but slight concentration-dependence the sedimentation coefficient of the urina protein suggests that the molecule is nea spherical, which, together with the low mo cular weight, renders it easily filtera through the glomeruli. The results of phy ical chemical examination of the prote except the thermal insolubility, are in go agreement with properties reported in lite ture for the classical Bence-Jones prote The difference in thermal behavior might

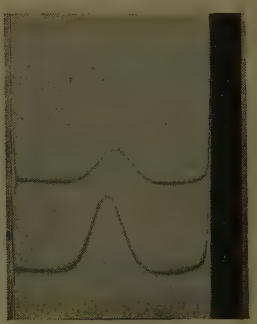

Fig. 2. Ultracentrifuge pattern of the urinary p tein 124 min. after reaching full speed.

ed by some differences in interchain
ls (Putnam, 1957). Rundles *et al.*
51) have published a case with similar
rophoretic mobilities of urine and serum
eins to those in our study. They give
es of 3.8 S for $S°_{20,w}$, $6.0 \times 10^{-7}$
/sec. for $D°_{20,w}$, and 60,700 for mole-
r weight of their classical Bence-Jones
ein.

risolia and Cohen (1953) have demon-
ed the existence of heterogenous mye-
globulins and have been able to sepa-
one of them into three components
h $S°_{20,w}$ 7.2, 17.7 and 26.2 respectively)
sedimentation. On the basis of electro-
esis and ultracentrifugation of serum in
case it appears likely that fraction M
ments mainly in the 7.5 S fraction as do
myeloma globulins in general, and to a
or extent in the macroglobulin fractions
S and 26.5 S.

## SUMMARY

he physical chemical properties of a
mal insoluble protein in the urine of a
ent with M-type multiple myeloma have
studied. Values of 3.93 S for $S°_{20,w}$,
$\times 10^{-7}$ cm²/sec. for $D°_{20,w}$ and 57,700
the molecular weight have been calcu-
l. pI was 5.6.

## ACKNOWLEDGEMENTS

institutional grant from the Sigrid Jusélius
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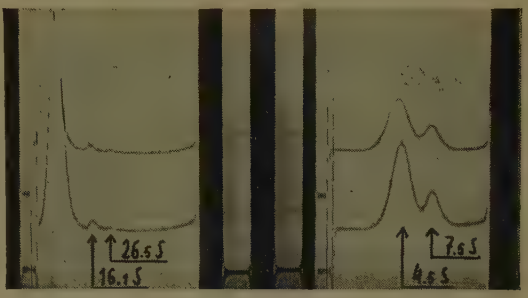


Fig. 3. Ultracentrifuge patterns of the serum 20 and 84 min. after reaching full speed. The components are indicated by their $S°_{20,w}$-values. Their relative concentrations, corrected for the Johnston-Ogston effect, are: 4.5S 67.3, 7.5S 30.0, 16.1S 2.2, and 26.5S 0.5 per cent.

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# TURNOVER OF PROTHROMBIN, FACTOR VII AND FACTOR IX IN A PATIENT WITH HEMOPHILIA A

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Clotting factors have a more rapid turnover than most other plasma proteins. This is often explained by assuming that coagulation is a continuous process in the normal body. However, a previous report from this laboratory (Hasselback & Hjort, 1960), showed that blocking of such hypothetical coagulation by a continuous infusion of heparin did not change the turnover rate of prothrombin and proconvertin (factor VII). The turnover was determined from the rate of disappearance of these factors from plasma after a large dose of Warfarin, which blocks their synthesis in the liver (Frick, 1958).

In this study we have used the same technique to determine the turnover of prothrombin, proconvertin and antihemophilia B factor (factor IX) in a patient with severe hemophilia A. For comparison, the experiment was repeated with identical technique in two healthy persons.

*Case report.*

The patient, S.B., was a 17-year-old male. His maternal grandfather and the grandfather's three brothers were bleeders. The son of the mother's first cousin is also a severe bleeder.

The bleeding tendency had been severe since infancy. Joint bleedings (ankles, knees, hips, bows) had been frequent and disabling; x-ray e mination of the knees showed marked hemoph arthrosis. In December 1954 a fracture of the femur led to his tenth admission to the Univers Clinic. The fracture healed very slowly, and stayed in the hospital for nearly a year. Dur this year, he had several periods with appare spontaneous joint and muscle bleedings.

The present experiment was performed in a g period. There was no anemia, and the clinical e mination revealed nothing abnormal, except for hemophilic joints. Table I gives a summary of "hemostatic tests". Similar results have been tained repeatedly over the last several years.

## MATERIALS

*Proconvertin reagent* was prepared by the me od of Hjort, (1957). It is an eluate of human ser containing proconvertin and Stuart factor (fac X), but less than 1 per cent of prothrombin. batch used contained about 30 per cent of Stu factor, as assayed in a one-stage system with Stu factor deficient plasma as substrate.

*Warfarin sodium.* Coumadin sodium (E Richmond Hill, N.Y.) for intravenous use was solved in distilled water to a concentration of 25 per ml.

The materials used for the clotting factor says are described in detail in the references g in the next section.

Table I. *"Hemostatic tests" in patient B.S.*

| Test | Patient B. S. | Normal | References |
|---|---|---|---|
| ole blood clotting time | 25–28½ min. | 2.7– 7.0 min. | Hjort & Stormorken (1957) |
| ding time | 4½–5½ » | 2 –12½ » | Hjort & Stormorken (1957) |
| elets per mm³ | 264 000 | 150 000–337 000 | Nygaard (1933) |
| halin time | 256 sec. | 57–64 sec. | Egeberg (1961) |
| k time | 17.5 » | 13–15 » | Quick system with human brain thromboplastin |
| onvertin | 58 % | 80–120 % | Aas (1952) |
| hrombin | 82 % | 80–120 % | See text. |
| ccelerin | 107 % | 80–120 % | Owren (1947) |
| hemophilia A factor | <1 % | 60–150 % | Egeberg (1961) |
| —»— B » | 73 % | 70–140 % | Egeberg (1961) |
| —»— C » | 84 % | 70–140 % | Egeberg (1961) |
| inogen | 260 mg % | 150–400 mg % | Jacobsson (1955), mod. by Blombäck & Blombäck (1956) |

## METHODS

*ollection of blood.* Venepuncture was performed a siliconized needle, and the initial 2—3 ml of d were discarded. Nine volumes of blood were collected directly into a plastic tube containing volume of 3.13 per cent w/v sodium citrate drate. This was mixed by gentle inversion and rifuged at 2,500 r.p.m. (ca. 1,800 G) for 30 ites at 4° C. The plasma was removed with a nized pipette and stored in plastic tubes at ° C. until assayed.

*ssay of clotting factors.* The plasma to be d was thawed at 37° C., transferred to an ice- , and tested within 30 minutes. All reagents e kept in icebath, except for the calcium ride and the calcium-proconvertin reagent mix- which were kept at 37° C., and the cephalin ension which was kept at room temperature. tests were carried out at 37° C., at least in icate. The clotting times were translated into cent of normal activity by means of correla- graphs (*i. e.*, dilution curves of our human dard plasma). See Hjort, Rapaport & Owren 5) for the dilution technique.

*ntihemophilia B factor* was assayed in a one- e cephalin system (Egeberg 1961). The follow- mixture was incubated in new glass test tubes 5 minutes: 0.2 ml cephalin suspension in optimal entration, 0.2 ml test plasma diluted 1:10, and ml plasma from a patient with severe hemo- a B. The mixture was then recalcified with 0.2 ml calcium chloride in optimal concentration, and the clotting time recorded.

*Proconvertin* was assayed in the system of Aas (1952): a mixture of 0.2 ml plasma from a patient with severe proconvertin deficiency, 0.2 ml human brain thromboplastin, and 0.2 ml test plasma diluted 1:10 was incubated for 3 minutes. It was then recalcified with 0.2 calcium chloride in optimal concentration, and the clotting time recorded.

*Prothrombin* was assayed in the system of Hjort *et al.* (1955) as modified by Hasselback & Hjort (1960). This system is specific for prothrombin. A mixture of 0.2 ml filtered, adsorbed ox plasma, 0.2 ml Russel viper venom in cephalin, and 0.2 ml test plasma diluted 1:50 was incubated for 3 minutes. It was then recalcified with 0.2 ml of a mixture containing 0.1 ml calcium chloride 70 mM and 0.1 ml proconvertin reagent, and the clotting time recorded.

## EXPERIMENTAL PROCEDURE

The patient was kept in bed on regular hospital diet. Blood was collected for a control specimen, and he then received 150 mg. Warfarin intravenously. Twenty-four and 48 hours later, he reiceived additional doses of 100 mg. At intervals, blood was collected for assay of the clotting factors. The plasma samples were frozen at —20° C., and all assays carried out when the experiment was finished.

After 72 hours the experiment was terminated,

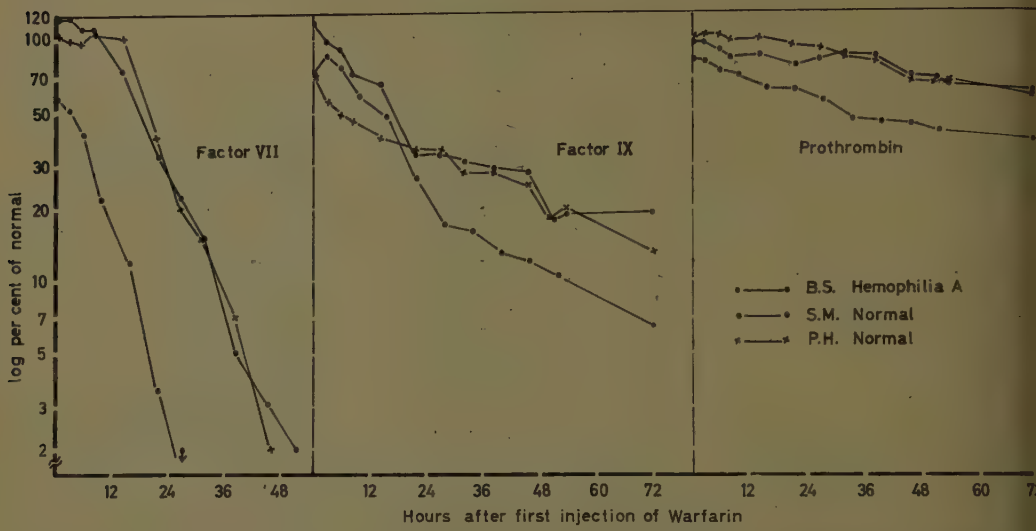


Fig. 1. The turnover of factor VII, factor IX and prothrombin in a patient with hemophilia A and in two healthy persons.

and he received 100 mg vitamin $K_1$ (Mephyton, Merck Sharp & Dohme, Rahway, N.J., U.S.A.) intravenously. He had no bleeding or untoward reactions during the experiment.

The same procedure was followed for the two normal persons, except that they were not kept in bed.

## RESULTS

Fig. 1 shows the results.

*Proconvertin* remained nearly unchanged for a period of 4-14 hours, and then dropped exponentially. The T/2 can be derived graphically from the straight part of the curve: 300 minutes for the patient and 290 and 320 minutes for the two normal persons.

*Antihemophilia B factor* decreased progressively, but the T/2 cannot be accurately determined from the curves. The best estimate appears to be about 24 hours in all three persons.

*Prothrombin* also decreased progressive but more slowly. The T/2 cannot be accu tely determined, but appears to be ab 3 days in all three persons.

## DISCUSSION

Frick (1958) and Hasselback & Hj (1960) have shown that the dose of W farin used in this experiment gives a m imum rate of fall of proconvertin and p thrombin in normal persons.

For proconvertin, the T/2-values ag with previous results in normals, *viz.*, 30 335 minutes (Hasselback & Hjort, 196 Similar results were reported by Loeli Esch, Cleton & Booij (1959). The lag ph showed greater variations in this experim (4—14 hours) than in the experiment ( 9 hours) reported by Hasselback & Hj (1960).

or the antihemophilia B factor, the litera- contains only few careful measurements. er a blocking dose of phenylindanedione, op (1958) found its turnover rate closer hat of proconvertin than to that of pro- mbin. Aggeler *et al.* (1956) and Bolton Clarke (1959) tranfused hemophilia B ents, and found that factor IX survived several days. Using an isotope technique normals, Adelson, Rheingold, Parker, ner & Kirby (1960) arrived at a T/2 of days.

or prothrombin, Borchgrevink, Egeberg, l, Skulason, Stormorken & Waaler 59) arrived at a T/2 of 2½—3 days, d on a blocking experiment with penylin- edione in a normal individual and on a sfusion experiment in a patient with an ated prothrombin deficiency. Hasselback Hjort (1960) found about 2½ days after rfarin, both in normals and in heparinized mals.

With this experimental technique, there- the turnover rate of these clotting fac- was not prolonged in our patient with re hemophilia. The data are conclusive proconvertin; for antihemophilia B fac- and for prothrombin some reservation ecessary since they have a slower turn- rate which makes it more difficult to rpret the curves.

ur findings support the conclusions of selback & Hjort (1960) that the rapid over rate of clotting factors is not caused continuous subclinical *in vivo* coagula- The present technique cannot rule out possibility that a smaller fraction turns as a consequence of coagulation, and results do therefore not disprove the ry of a continuous coagulation process in normal individuals. However, the rapid turnover rate of clotting factors should not be used as evidence for such a process.

## SUMMARY

The disappearance of prothrombin, proconvertin (factor VII) and antihemophilia B factor (factor IX) from plasma after blocking of the liver synthesis by large doses of Warfarin reflects the *in vivo* turnover of these factors. By this technique, the turnover was investigated in a patient with severe hemophilia A and in two healthy persons.

The turnover of proconvertin was normal in the patient. It was probably also normal for prothrombin and the antihemophilia B factor, but these data are less conclusive. These observations give additional support to the view that the rapid turnover of these factors is not caused by continuous *in vivo* coagulation.

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# VISCOMETRIC DETERMINATION OF HYALURONIDASE ACTIVITY IN BIOLOGICAL FLUIDS

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he work of previous authors gives ence that the most favorable conditions the viscometric determination of hyal- idase activity in biological fluids are d in an acid milieu of low ionic strength an ample dilution of the samples (Gi- , 1955, and v. Berlepsch, 1958).

he present method complies with these irements, and some improvements in the gn of the viscometers and in the pro- re have been made to increase efficiency to economise in materials.

e have given special attention to the tics of the degradation of hyaluronic and have been able to distinguish be- n two different kinds of viscosity- cing agents. The presence of inhibitors be seen both from the shape of the de- ation curve and from the effect of ing the samples.

he method has been used for studies on rent hyaluronic acid degrading agents in serum, tissue extracts and urine from normal subjects of different species.

## METHODS

### *Apparatus.*

*The viscometers,* manufactured to order by F. C. Jacob, Copenhagen, are of Ostwalds type and similar to those described by Dalgaard-Mikkelsen and Kvorning, 1948. Improved details in the design appear from Fig. 1. A resting place has been provided for solid particles at the bottom of the wide tube, and the upper part of the capillary around the reservoir has been made wider (1 mm bore) than the rest of the capillary (0.31 mm bore). This greatly facilitates the handling and also the observation of the meniscus at the same time as it diminishes the influence of surface tension. Last but not least it makes it possible to perform the mixing of enzyme and substrate solutions in the viscometer after thermal equilibration. Flow time is about 30 sec. for water (38° C) and 150 µl suffices for the filling. Four viscometers are mounted in a row in a perspex bar which is rested as an hypotenuse over two adjacent sides of a glass thermostat (38.00° C ± 0.02). An excentric, conical sleeve of polythene permits a firm fixation of the viscometer in the holes irrespective of small variations in the distance between the two branches.

In between two experiments the viscometers are washed *in situ,* first with 3 per cent saline, then

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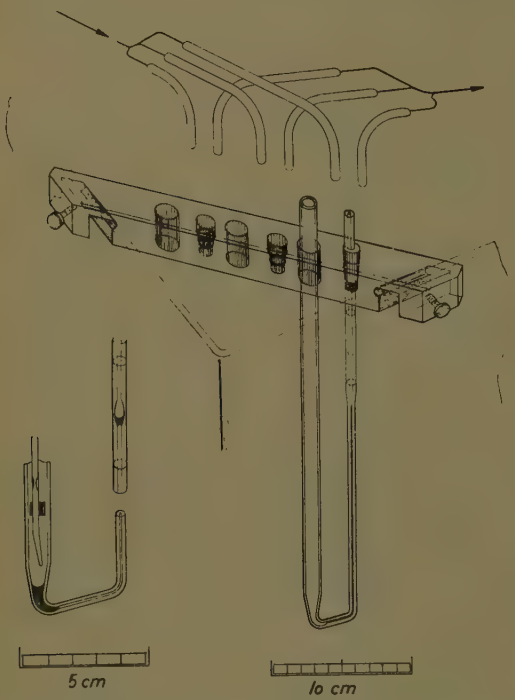



Fig. 1. One viscometer in the perspex holder for three viscometers. Detailed drawing of the placing of sample and substrate. Description in text.

with a flow of redistilled water for two minutes, and finally with 93 per cent w/w ethanol to facilitate the subsequent drying with an air stream. Over night they were left with chromic acid, and before the measurements all the viscometers are rinsed with a continous flow of 3 per cent saline for three hours. 3 per cent saline supply and suction are arranged through soft PVC tubings from branched glass tubes to all the viscometers at the same time, and with another row of four viscometers just behind the first one, measurements can be made in one set while the other set is being rinsed.

*Constriction pipettes* of the Carlsberg Laboratory type (125 µl, 25 µl) are used and are rinsed in a similar way. A small plastic gadget near the tip of the 125 µl pipette prevents scratching of the glass tube and untimely emptying of the pipette when it is lowered down the wide tube to place the substrate as indicated on Fig. 1. A thin polythene tubi (Tolcothene sleeving, 0.5 mm bore, 15 cm long) connected to the tip of the filled 25 µl pipette order to place the sample in the bulb as indicated Fig. 1. The liquid must flow gently to avoid lo and it is convenient to draw out the tubing in small flame and cut off pieces of the narrow pa until the resistance is suitable. Fresh tubing is us every day, and it is cleaned together with the co striction pipette.

## *Materials.*

*Buffer.* 0.15 M acetic acid — sodium aceta buffer of pH = 4.63 is used, (0.072 M sodiu acetate, 0.078 M acetic acid). 5 ml ampoules a autoclaved and stored at 4° C until use.

*Albumin.* Highly purified albumin from norm human serum is prepared by dr. phil. Albert Ha sen, Statens Seruminstitut, Copenhagen, and kind presented to us; less purified albumin often contai some viscosity-reducing activity. 10 mg are di solved in 10 ml of buffer by gently tilting the tu The solution is made afresh every day and is us for the preparation of substrate and sample sol tions.

*Hyaluronic acid (H.A.)* is a freeze dried prepar tion from bovine synovial fluid, kindly presented us by Dr. J. Ploug, Leo Pharmaceutical Produc Ltd., Copenhagen. 7.5 mg are added to 3 ml buffer + albumin. After one to two hours at 38° the somewhat milky mixture is shaken and cent fuged at 6000 g for 20 minutes. The supernata is now opalescent and keeps a constant viscos during the days work.

*Hyaluronidase* U.S.P. reference standard is d solved in buffer + albumin. Foaming is avoided hyaluronidase activity can be removed from t solution with the foam. (If foaming should occ however, full activity can be regained, when t foam is destroyed by centrifugation.) Even wh kept at room temperature the activity is const during the day.

*Lysozyme,* a three times recrystallized prepa tion from egg-white (Sigma) is used. Usually 3 of lysozyme is dissolved in 1000 µl of buffer albumin. When kept at room temperature a sligh increasing activity is found during the day.

*Serum.* Dilutions of 30 µl in 1000 µl of buffer + bumin are made. Storage of undiluted serum for week or so at 4° C does not influence the activity. ilutions are prepared immediately before use, cause most serum dilutions show a slight in- ease in activity during the day when kept at room mperature.

*Tissue extracts.* 3 mg of tissue are added to 100 of buffer + albumin, and given a mild and termittent treatment in a Potter Elvehjem ho- ogenizer, frozen at —23° C, thawed, mixed, centri- ged at 6000 g for 20 minutes and kept in a stop- red test tube at 4° C until use. When treated in is way only little or no variation in total viscosity- ducing activity is recorded by repeated determina- ons during the day. The final dilution of tissue xtract as of serum in the reaction mixture is 206 nes.

*Urine.* When kept at room temperature, no varia- on in activity during the day is recorded in samples om normal subjects, whether the samples are luted with buffer + albumin or not.

*rocedure.*

125 µl of substrate solution are placed in the ide tube without contaminating the wall. A 25 µl mple of the solution to be tested is placed in the lb (compare Fig. 1). Three more viscometers are ade ready in the same way while the first one is ft to come into temperature equilibrium with the ermostat. A small pressure is then applied to ow the sample down to the substrate and five air bbles are blown through. To complete the mixing e liquid is then sucked up in the bulb and blown wn. Immediately afterwards, the liquid is sucked slightly over the upper mark and a first reading the flow time can be made. The three other scometers are started in the same way while the uid in the first one is flowing down. Two stop- tches are in use for each viscometer, one for the w-time determination and one for reading time reaction, starting from the moment of mixing. serial measurements one might prefer to per- m mixings and flow-time determinations after a ndardized schedule for the four viscometers. The action is followed for at least 25 minutes, often nger. Flow time is determined every minute and half for each viscometer.

At least once a day, flow-time for redestilled water is controlled and repeated determinations are made on blanks (125 µl of substrate + 25 µl of buffer + albumin). Flow-time for these blanks proves to be constant and reproducible, and serves as initial flow-time value for the determinations made on the same day and with the same viscometer.

Flow-time is plotted against reaction time.

## RESULTS

*Evaluation of results.*

Total viscosity-reducing activity was expressed in the usual arbitrary units (a. u.): per cent decrease in specific viscosity after 20 minutes reaction time, calculated as

$$\text{a.u.} = 100\,\frac{\text{initial flow time} - \text{20 min. flow time}}{\text{initial flow time} - \text{water flow time.}}$$

A study of the shape of the reaction curves, however, made it possible to distinguish two different viscosity-reducing factors, one fast reacting factor which had finished its action within the first minute, and another more like an ordinary enzyme reaction. The action of the fast reacting factor could be imitated for instance by lysozyme, hence we will refer to it as the "L-factor" in contrast to the "H-factor", the action of which could be imitated by standard hyaluronidase with or without the addition of various inhibitors (Fig. 2).

In sufficiently dilute samples the action of lysozyme and of standard hyaluronidase seems to be approximately additive. When biological samples give a curve like Fig. 3, we therefore tentatively describe it as a mixture of L- and H-factor with so many a. u. of L-factor as corresponds to an extrapolation of the curve to zero time and an

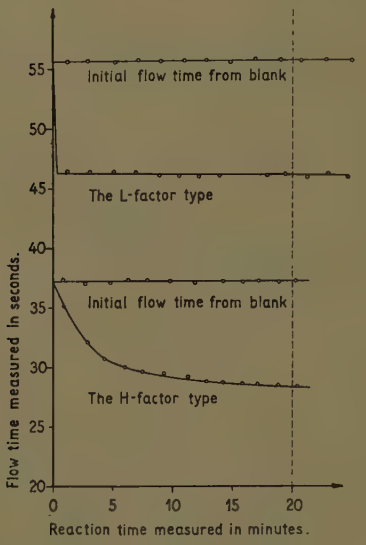

Initial flow time from blank
The L-factor type
Initial flow time from blank
The H-factor type
Flow time measured in seconds.
Reaction time measured in minutes.

Fig. 2. Examples of reaction curves of the L-factor and H-factor types. The upper curve is from an experiment with undiluted rabbit serum in viscometer No. 5, which has a water flow time of 38.5 sec., and the lower curve is from another experiment with a tissue extract from a dog lymph node in viscometer No. 3 (water flow time 26.5 sec.). Tissue extract and substrates were prepared as described above.

amount of H-factor activity which corresponds to the rest of the a. u.'s found from the 20 min. reading.

*The accuracy of the method.*

*The effect of albumin.*

The water flow time determination (average of ten readings), from day to day was constant, in seconds for viscometers 1, 2, 3 and 4 during 20 consecutive days: 28.0, 28.1, 26.5 and 25.5. The relative visosity of 125 $\mu$l of the substrate (2.5 g/l of hyaluronic acid in buffer + alb.) + 25 $\mu$l buffer + alb. was 1.440. The values varied ± 2—3 per cent on different days, but on the same day no larger difference than 0.5 per cent was found between values from individual viscometers.

The standard deviation in triple determinations on 9 samples of increasing standard hyaluronidase activity was 1.0 a.u (a. range: 5—80 a. u.); and in 30 duplicate determinations on extracts of human kidney cortex the standard deviation was 0.9 a. (a. u. range: 23—72 a. u.).

Particularly with the dilute standard hyaluronidase solutions it was a condition for obtaining this degree of reproducibili

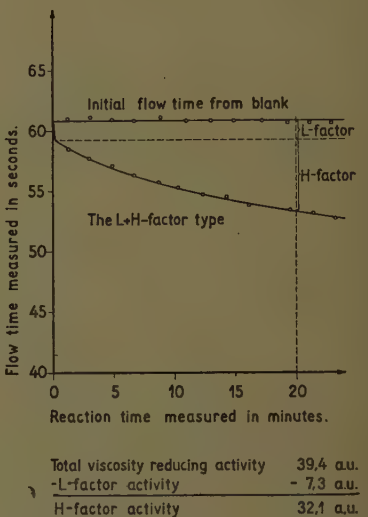
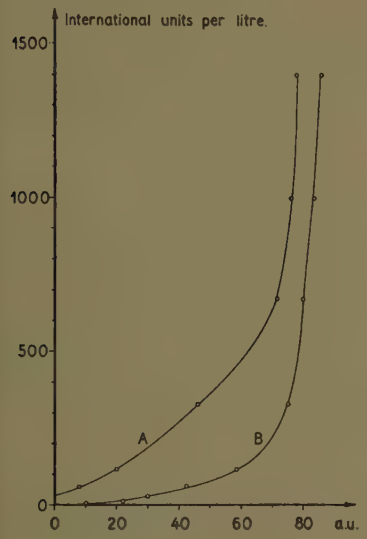



Fig. 3. Example of a reaction curve revealing mixture of H- and L-factor. Serum from a healt male blood donor, diluted and tested according our standard procedure as described above (visc meter No. 7, water flow-time 41.5 sec.). The calcul tion of the actual a.u.'s of H- and L-factor activiti is shown under the curves. The total viscosit reducing activity is calculated in a.u. as described the text. The L-factor activity is convenient estimated in a similar way after 20 min.'s reacti time, although the reaction is completed before t first viscometric determination can be performe The H-factor activity is then calculated as a diffe ence.




g. 4. Calibration curves. Activity of U.S.P. aluronidase reference standard in arbitrary units u.) is plotted against different weight concentrans expressed in international units per litre of action mixture. Curve A, when no albumin is ded and curve B when 1.0 g/l of pure albumin d been added both to sample and to substrate. rve B is used for the conversion of H-factor ivities in a.u. to standard hyaluronidase concentrations in international units per litre.

at albumin had been added in the way scribed above. Without albumin the activ- in a. u. is much smaller (*cf.* Fig. 4), and standard deviation is about six times ger. The exact concentration of albumin not critical, as calibration curves quite nilar to curve B in Fig. 4 are obtained h half or double the amount of albumin, also Table I, d., e., and f.).

The addition of albumin was actually induced after it had been found that the ndard deviation was much smaller with um dilutions than with solutions of the pure enzyme. Table I reports a characteristic example of a set of duplicate experiments which led to the actual choice.

Table I. *Effect of albumin.*

*Activity in arbitrary units of USP hyaluronidase reference standard in the concentration of 55.6 international units per litre of reaction mixture at pH 4.63.*

| | Conc. of albumin in reaction mixture in g/l | Sequence of mixing the components. | Arbitrary units |
|---|---|---|---|
| a | 0 | — | 7.5—8.8 |
| b | 0.17 | 1. Enzyme dissolved in buffer. 2. Addition of albumin to 1. No albumin in substrate mixture | 17.8—17.3 |
| c | 0.17 | 1. Albumin dissolved in buffer. 2. Addition of enzyme to 1. No albumin in substrate mixture. | 28.6—28.8 |
| d | 1.0 | 1. Albumin dissolved in buffer. 2. Preparation of substrate and enzyme solution with 1. | 39.9—40.0 |
| e | 0.5 | Like d. | 39.2—39.3 |
| f | 2.0 | Like d. | 39.6—40.1 |

A. The effect of albumin is shown (*cf.* a and d). B. It is shown that the sequence of mixing buffer, albumin and enzyme is of importance (*cf.* b and c). C. It is shown that the concentration of 1 g/l of albumin in the reaction mixture is not critical, when substrate and enzyme solution are made as described under materials (*cf. d, e, and f.*).

These results may seem to corroborate the assumption, made by several authors, that hyaluronidase preparations contain inhibitory substances. As the presence of protein is known to make it keep its activity better under storage in solution (*cf.* Alburn and Whitley, 1951, and Mathews and Dorfman, 1955) there is also the possibility that enzyme molecules when unprotected by other proteins may aggregate to less active and less stable multiples, and as it seems, particularly so during the process of dissolution (Table I, a, b, and c.).

The activity of lysozyme seems not to be influenced by the presence or absence of albumin.

*The substrate.*

*Hyaluronic acid.* Practically all our experiments over two years have been made with synovial hyaluronic acid from one single batch. A series of experiments with another batch of a somewhat lower specific viscosity gave very similar results both with respect to H-factor and L-factor activities.

Two preparations of hyaluronic acid from umbilical cords behaved differently. One of these preparations was made at Statens Seruminstitut by the usual procedure and the other was a specially purified sample completely free of protein and of chondroitin sulphate, purified by Drs. Marcker and Berthelsen by an ion exchange procedure. Both preparations had a high molecular weight and were used in such low concenration (1.0 g/l) that they gave about the same viscosity as the 2.5 g/l solutions of the synovial preparation. At first these substrates seemed unimpaired by both hyaluronidase and lysozyme, and only aft raising the hyaluronidase concentrati (1000 times with the protein-free substrat did we obtain similar degradations as tho reported with the synovial fluid prepar tions[1].

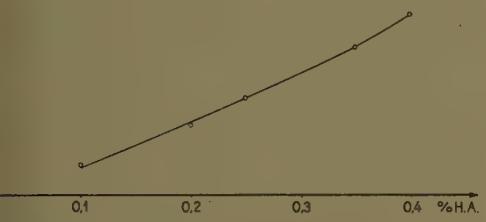
When biological fluids containing visc sity-reducing agents were added to the tw umbilical cord hyaluronic acid substrates activity could be detected at all with t protein-free preparation, and only slight, b significant, H- and L-factor activities cou be detected with the other.

These results raise some interesting ques ions to be answered by further experiment but so far it is only possible to conclude th the synovial fluid preparations should preferred for the detection of small enzym activities.

The addition of albumin (1.0 g/l) to t protein-free umbilical cord hyaluronic ac preparation had no effect on the absence activity when biological fluids were mea ured; the appearance of the substrate, ho ever, changed from a clear solution to t opalescent mixture, which was always fou with the other umbilical cord preparati and the synovial fluid preparation used the present method.

This opalescence of the substrate m raise some doubt as to whether it is rea well defined, so we investigated the influen of the concentration of hyaluronic acid

[1] This seems in concordance with Meyer, Ho man & Linker, 1960, who report that preparati of hyaluronate obtained from umbilical cord found to require ten times the amount of hya ronidase to reach half-turbidity as compared t standard prepared from human mesenthelioma.




r. 5. The relation between concentration of luronic acid (H.A.) in the opalescent substrate the relative viscosity ($\eta_r$). (Solvent: 0.15 M tic acid-sodium-acetate buffer, pH 4.63 with 1.0 g/l of pure albumin.)

relative viscosity and were satisfied to d a practically linear dependence (Fig. 5). Variation of the substrate concentration m 1.0 to 4.0 g/l in buffer + alb. did not er the measured activities much — one or o per cent were the largest variations — en large activities were measured.

*Electrolytes in buffer.* The acetic acid-tate buffer was chosen since it turned out t larger activities were found with this ffer than with a citric acid phosphate ffer of the same pH (4.6). At pH about n citric acid-phosphate or in barbiturate ffers, standard hyaluronidase had only ut a third of the effect, and no effect at could be found of lysozyme or of the ious biological fluids.

Chloride ions have often been reported to luence the activity of hyaluronidase and Fig. 6 we report a series of experiments constant ionic strength and constant pH 4.6, where the acetic acid-acetate is grad-ly replaced by sodium chloride. It is note-rthy that lysozyme activity disappears npletely when acetate is absent, whereas hyaluronidase activity increases by some ten to twenty per cent.

The appearance of the substrate also changes as chloride replaces acetate from more opalescent through less opalescent to a practically clear solution when the acetate content is small. Relative viscosity increases at the same time.

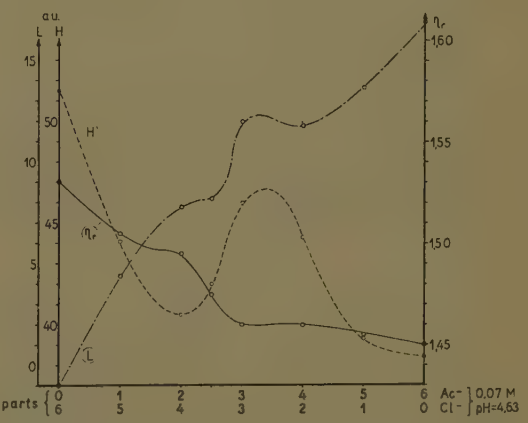



Fig. 6. *Dependence of the activity of lysozyme and standard hyaluronidase and the relative viscosity of the substrate upon the composition of acetate and chloride in buffer pH 4.63.*

L: activity in a.u. of 0.5 g/l of lysozyme in the reaction mixture. H: activity in a.u. of U.S.P. hyaluronidase reference standard per litre reaction mixture. $\eta_r$: relative viscosity of the substrate. Like the enzyme concentrations, ionic strength, pH, hyaluronic acid concentration and albumin concentration are kept constant during the experiment.

L., H. and $\eta_r$ are determined when 0.07 M sodium acetate (0.15 M acetic acid — sodium acetate buffer pH 4.63) is stepwise replaced by 0.07 M sodium chloride (adjusted to pH 4.63 with hydrochloric acid) in the buffers used for preparation of substrate and enzyme solutions. Every determination is made exactly 45 minutes after preparation of enzyme solution. All determinations are made on the same day, and the whole experiment has been performed 3 times with similar results.

Small amounts of chloride ions have no significant effect in either of the measured qualities and we feel justified in neglecting the influence of the varied contents of the biological fluids since they will always be mixed into a much larger volume of acetate buffer, (*e. g.* the 206-fold dilution of serum, tissue extract and urine).

*H-factor inhibition in serum and urine. The effect of dilution.*

By means of the calibration curve B in Fig. 4 it should be possible to calculate the actual amount of active enzyme present from the measured number of arbitrary units, provided that the H-factor is identical with the standard hyaluronidase or at least that follows the same dilution curve. It is w known that inhibitors are present in seru and that their effect can be suppressed dilution (Gibian, 1955). We have found th the same applies to urine. Some of our exp rimental results are recorded in Tables II a III. When further dilution gives no furth increase in activities converted to conce trations of standard hyaluronidase, we thi that inhibition is totally quenched.

The dilution which we have chosen f the routine determinations in serum (1:2 that is 30 µl of serum to 1 ml of buffer albumin, further diluted 25:150 in the action mixture) brings the activity with

Table II: *Elimination of H-factor inhibition in serum by dilution. 6 dilutions with buffer + alb. of two sera A. and B., obtained from two healthy female blood donors.*

| 1. Dilution | 2. Total (a.u) | | 3. L(a.u.) | | 4. H(a.u) | | 5. int.U. per litre | | 6. int.U. per ml of serum | |
|---|---|---|---|---|---|---|---|---|---|---|
| | A. | B. | A. | B. | A. | B. | A. | B. | A. | B. |
| 1:6 | 67.9 | 69.8 | 25.5 | 26.1 | 42.4 | 43.7 | 60 | 62 | 0.4 | 0.4 |
| 1:16 | 78.0 | 80.3 | 27.2 | 28.6 | 50.8 | 51.7 | 90 | 90 | 1.4 | 1.4 |
| 1:26 | 73.7 | 76.4 | 15.3 | 21.4 | 58.4 | 55.0 | 125 | 110 | 3.2 | 2.9 |
| 1:106 | 50.1 | 49.4 | 7.5 | 6.9 | 42.6 | 42.5 | 60 | 60 | 6.4 | 6.4 |
| 1:206 | 33.0 | 38.9 | 3.4 | 8.9 | 29.6 | 30.0 | 30 | 30 | 6.2 | 6.2 |
| 1:406 | 23.3 | 25.8 | 0.9 | 5.2 | 22.4 | 20.6 | 16 | 15 | 6.4 | 6.1 |

1. Volume of dilution over volume of serum in the reaction mixture; 2. Total viscosity reducing activities in a.u. (average of two determinations); 3. Estimated L-factor activities in a.u. (*cf.* Fig. 3); 4. Estimated H-factor activities in a.u. (*cf.* Fig. 3); 5. H-factor activities converted to equivalent amounts of standard hyaluronidase in international units per litre of reaction mixture (by means of curve B in Fig. 4); 6. Equivalent amounts of standard hyaluronidase in international units per ml of serum (0.001 times the product of the numbers from columns 1 and 5.). Column 6 shows that H-factor inhibition is quenched by a 106-fold dilution of the samples since further dilution gives no further increase in international units per ml of serum.

range of optimal accuracy of the method l also within the range where inhibition ms completely eliminated.

Conditions for the urine determination are s favorable. The activities of the undiluted ne samples vary from about 5 a. u. to ximally 50 a. u. The shape of the reaction ves already reveals that strong inhibition st be present, since they are more rect- ear than those of the standard hyal- nidase solutions of comparable activity. ution experiments corroborate this con-

Table III. *Suppression of H-factor inhibition in urine by dilution.*

*dilutions with buffer + alb. of two night ne samples: A., obtained from an adult female and B., obtained from an adult normal man.*

| 1 lution | 2. H-factor a. u. | | 3. int.U.per litre | | 4. int.U.per ml of Urine | |
|---|---|---|---|---|---|---|
| | A. | B. | A. | B. | A. | B. |
| :6 | – | 49.0 | – | 82 | – | 0.5 |
| :10 | 43.5 | – | 63 | – | 0.6 | – |
| :26 | 41.0 | 21.4 | 56 | 18 | 1.5 | 0.5 |
| :106 | 21.8 | 13.0 | 18 | 8 | 1.9 | 0.8 |
| :206 | 18.2 | 12.3 | 12 | 7 | 2.5 | 1.4 |
| :306 | 15.5 | 10.2 | 11 | 6 | 3.4 | 1.8 |
| :406 | 13.8 | 10.5 | 8 | 6 | 3.2 | 2.4 |

Volume of dilution over volume of urine sample the reaction mixture. 2. H-factor activity in trary units (a.u.), average of two determina- s. 3. H-factor activities converted to equivalent unts of standard hyaluronidase in international s per litre of reaction mixture (by means of ve B in Fig. 4.). 4. Equivalent amounts of dard hyaluronidase in international units per ml rine (0.001 times the products of the numbers in column 1 and 3).

nple B. seems to contain more inhibitor than sample A.

clusion as shown in Table III. The urine samples seem to require at least as much dilution as the serum samples before inhibition is suppressed.

When maximal H-factor activity (about 50 a. u.) is found in undiluted urine samples, a dilution of 300—400 times still gives activities which can be determined with adequate accuracy, but when urine samples with lower H-factor activity are measured, we get such low activities, when proper dilution of the samples are made, that they become too small to be detected by the present method. So separation of H-factor from H-factor inhibition and concentration of the pure H-factor in urine is necessary before adequate work on urinary excretion of H-factor (hyaluronidase) can be done.

In view of these facts it is not surprising that the literature contains contradictory statements on the variation of hyaluronidase activity with urine volume/min. (Ginetzinsky 1958, Ivanova 1958, Berlyne 1960 and Dicker & Eggleton 1960). In the various methods urine is diluted, dialyzed and concentrated in different ways, and how enzyme and inhibitors behave in these circumstances is, of course, quite uncertain.

## SUMMARY

1. Minor changes in the design of Ostwald viscometers have been made in order to make viscometric determinations of hyaluronidase quicker and easier, more sensitive to small activities and more economical in sample and substrate volume without loss of accuracy.

2. For the reproducible determination of hyaluronidase activity in biological fluids it

is important to work in an acid buffer of low ionic strength and to use diluted samples.

3. Calibration curves with USP hyaluronidase reference standard are reported. Addition of albumin increases both the activity itself and the reproducibility of the measurements.

4. The importance of studying the shape of the degradation curves is stressed. It has been possible in biological fluids to distinguish two different agents which decrease the viscosity of hyaluronate, named H-factor and L-factor.

5. When viscosity-reducing activities such as those found in biological fluids are to be measured, hyaluronate prepared from synovial fluid is preferable to that prepared from umbilical cords.

6. Dilution experiments reveal that H-factor inhibition occurs in urine from normal human beings. It is suggested that this explains controversies in the literature.

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# STUDIES ON THE DETERMINATION OF HUMAN CHORIONIC GONADOTROPHIN BY COMPLEMENT FIXATION

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In earlier reports (Brody & Carlström 960, 1961) we have presented a serological ethod for the quantitative determination of uman chorionic gonadotrophin (HCG). We ere demonstrated the capacity of HCG to duce in rabbits the formation of comple-ent-fixing antibodies against the hormone. Vith the rabbit antisera the quantitative etermination of HCG in serum from regnant women was possible with the mplement-fixation test. As methodological roblems have earlier been only briefly out-ned, we intend here to give a more detailed eport of the complement-fixation test adapt-l to the qualitative and quantitative ana-sis of HCG.

## MATERIALS AND METHODS

*Antigen.* As antigen we have used a commercial eparation of HCG (Gonadex), manufactured by B Leo, Hälsingborg, Sweden. It is claimed to a highly purified substance, containing 6,000 I.U. r mg. This preparation has been used for pre-ring and testing the HCG-antisera. In the com-ement-fixation tests it was checked against the ternational standard preparation of HCG.

*Preparation of antisera.* For inoculations HCG was used either dissolved in physiological saline or incorporated into an adjuvant. The antigen-adjuvant mixture was prepared with 8.5 parts paraffin oil, 1.5 parts Arlacel A (Atlas Powder Co., Wilmington, Delaware) and 10 parts physiological saline containing the hormone. The oil and emulsifier were first mixed, then the hormone solution was added in small portions and the mixture shaken until a paste-like emulsion was obtained. For part of the inoculations *Mycobacterium butyricum* was added to the adjuvant. The bacteria were heat-inactivated and mixed into the oil before addition of the emulsifier and hormone solution. The concentration was 0.1 mg of mycobacteria per ml of adjuvant.

Male and female adult rabbits were used in this study. Four different immunization schemes were tried. *Group I:* four animals received 4 ml of HCG in saline (12,000 I.U.) injected into the foot pads. Subsequently 1 ml of HCG in saline (3,000 I.U.) was given by the same route once a week for 3 weeks. Blood was withdrawn by cardiac puncture 8 days after the last injection. *Group 2:* four animals received initially 1 ml of HCG in saline (3,000 I.U.) injected into one foot pad and simultaneously 4 ml of HCG-adjuvant mixture (6,000 I.U.) without mycobacteria subcutaneously. Subsequently these rabbits received 8 foot pad injections of 1 ml of HCG in saline (3,000 I.U.) on

alternate days and an additional similar foot pad inoculation after one week. Blood was withdrawn after 8 days. *Group 3:* four rabbits received 1 ml of HCG in saline (600 I.U.) intraperitoneally. At the same time and on 5 further occasions at intervals of one to two months 2 ml of HCG-adjuvant mixture without mycobacteria were given subcutaneously. The HCG-content of the inocula was 600 I.U. the first three times, thereafter 1,200, 6,000 and 9,000 I.U. Blood was drawn at various intervals during this period, usually one to three weeks after each injection. *Group 4:* twelve rabbits were immunized each with 20 ml of HCG-adjuvant mixture (12,500 I.U.) containing mycobacteria. The mixture was given at four sites subcutaneously and intramuscularly. This was repeated twice at intervals of 3 weeks. A booster injection of HCG in saline (3,000 I.U.) was given intravenously 4 to 6 weeks after the last inoculation. Blood was withdrawn by cardiac puncture one week after the booster injection. In some animals a similar booster was given after another 4 to 6 weeks and blood withdrawn after an additional week.

Blood was collected under sterile conditions with dry needles and syringes and transferred to centrifuge tubes covered with rubber caps. It was allowed to stand at room temperature for 3 to 5 hours, then the serum was separated by centrifugation, heated at 56° C for 30 minutes and stored, without preservative, at —20° C until used.

*Diluent.* Veronal buffer was used for dilutions. A stock solution was prepared by dissolving 25.5 g of sodium 5,5-diethyl barbiturate in 2 liters of glass-distilled water. This was mixed with a solution containing 210 g of NaCl in the same medium. To this was added 4.20 g of $MgCl_2 \times 6H_2O$, 0.93 g of $CaCl_2 \times 2H_2O$ and 78.2 ml of 1 *N* HCl. The solution was made up to 5 liters, the pH brought to 7.4—7.5 by 1 *N* HCl or NaOH, the solution sterilized, the pH-value checked, and the buffer stored at room temperature. For further use this stock solution was diluted 1:5 and kept at 4° C.

*Hemolytic system.* Sheep blood was withdrawn under sterile conditions, mixed with an equal volume of Alsever's solution (containing 4.10 g of glucose, 1.60 g of sodium citrate, 0.84 g of NaCl and 0.11 g of citric acid per 200 ml of solution), filtered through a Jena glass filter No. G 5) and stored in aliquots at 4° C. Immediately before use the sheep-blood suspension was centrifuged at 1,200 r.p.m. for 10 minutes. The supernate was withdrawn and the red blood cells were washed 3 to 4 times with chilled veronal buffer. An aliquot of the lightly packed cell suspension was diluted 1:50 with chilled veronal buffer. After thorough mixing a 1 ml sample was taken and diluted to 10 ml with water which resulted in lysis of the erythrocytes. Optical density (O.D.) of the lysate was determined at 560 mμ with a Zeiss spectrophotometer and square glass cuvettes having a 1-cm light path, water serving as the blank. The O.D. obtained was used to calculate the amount of veronal buffer required to adjust the red blood-cell suspension to the desired concentration. Our standard red blood-cell suspension has an O.D. of 0.740 which approximately corresponds to a 0.4 per cent blood-cell suspension.

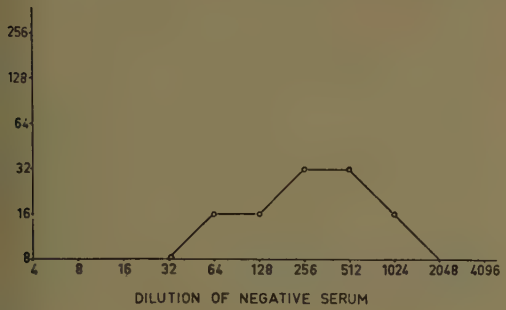
The anti-sheep-erythrocyte rabbit serum (obtained from the State Bacteriological Laboratory, Stockholm) was mixed with an equal amount of sterile glycerin. It was stored at —10° C. Six minimum hemolytic doses of amboceptor were used.

As complement the same batch of pooled guinea-pig serum was employed throughout the study. It was stored in sealed ampules at —70° C. Two complement units were used in the tests.

*Specimens to be tested for HCG.* Blood was examined from pregnant and non-pregnant women. The blood samples were collected under sterile conditions, the serum separated by centrifugation after 1 to 24 hours and inactivated at 56° C for 30 minutes. All samples were stored at —20° C.

*Complement-fixation test.* The plate technique of Fulton & Dumbell (1949) as modified by Svedmyr, Enders & Holloway (1952) was employed. Antigen (Gonadex[1], international standard preparation of HCG[1] or specimens to be tested for HCG), anti-

[1] The HCG-preparations were dissolved in veronal buffer or in sera from non-pregnant women.




ig. 1. Two-dimensional titration of HCG-anti-rum with serum from a non-pregnant woman. he greatest antiserum dilution inhibiting hemo-lysis is indicated in the figure.

rum, and complement were incubated for approx-nately 18 hours at 4° C. The sensitized sheep cells ere then added and readings taken after incuba-on at 37° C for 2 hours. To rule out anticomple-entary effects each component in the tests (anti-en, antiserum, and diluent) was checked by com-ement titration. Specimens displaying anticomple-entary effects were treated according to Taran 956) and discarded if the treatment was un-ccessful.

*Statistical methods.* Statistical analysis of the curacy of the quantitative serological determina-on procedure for HCG was performed according conventional methods (Snedecor, 1957).

## RESULTS

*Comparison between effects of various chniques of immunization.* In order to ex-nine the presence of complement-fixing ntibodies in the rabbit sera these were tested two-dimensional titrations with a standard olution of HCG, dissolved in veronal buffer id containing 1,500 I.U. of the hormone per l. The antibody titers are given as the ciprocal of greatest dilution of antiserum mpletely inhibiting hemolysis at optimal ntigen dilution.

Sera from animals in groups 1 and 2 did not contain any measurable amount of complement-fixing antibodies.

Comparatively low-titer antisera were obtained from animals in group 3. These rabbits were immunized for up to 9 months. In spite of repeated inoculations no rise in antibody titer was, however, observed after the fourth month. The antibody titers in this group varied from 16 to 64.

Animals in group 4 yielded sera with high antibody titers. An additional intravenous booster 4 to 6 weeks after the first booster did not cause any change in titer. The antibody titers varied from 64 to 2048, most of them being 256 or 512.

Rabbit sera obtained before immunization did not contain any complement-fixing antibodies against HCG.

These results indicate that immunization according to scheme 4 is preferable. In the experiments to be reported below we have employed exclusively antisera prepared according to this method.

*Reactions between HCG-antisera and specimen from non-pregnant women*[1]. Quite a few antisera fixed complement in the presence of negative sera. These reactions occurred in a characteristic zone of serum dilution and in low antiserum dilutions. Fig. 1 represents a two-dimensional titration with negative serum illustrating these observations. As is seen, there are no positive reactions in the area of low serum dilutions.

On the assumption that this complement

[1] Specimens from non-pregnant women will be referred to as negative, and from pregnant women as positive.

Table I. *Two-dimensional titrations of unabsorbed and absorbed HCG- antiserum and negative serum.*

| Dilution of negative serum | Serum/antiserum ratio | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 Dilution of antiserum | | | | 1:8 Dilution of antiserum | | | | 1:4 Dilution of antiserum | | | |
| | 8 | 16 | 32 | 64 | 8 | 16 | 32 | 64 | 8 | 16 | 32 | 64 |
| 4 | − | − | − | − | − | − | − | − | − | − | − | − |
| 8 | − | − | − | − | − | − | − | − | − | − | − | − |
| 16 | − | − | − | − | − | − | − | − | − | − | − | − |
| 32 | + | − | − | − | − | − | − | − | − | − | − | − |
| 64 | + | + | − | − | − | − | − | − | − | − | − | − |
| 128 | + | + | − | − | + | − | − | − | − | − | − | − |
| 256 | + | + | + | − | + | − | − | − | − | − | − | − |
| 512 | + | + | + | − | + | + | − | − | − | − | − | − |
| 1.024 | + | + | − | − | + | + | − | − | − | − | − | − |
| 2.048 | − | − | − | − | + | + | − | − | − | − | − | − |

+ indicates no hemolysis.
— indicates complete hemolysis.
In the narrow region between complete and no hemolysis + is for simplicity used to indicate ≦ 50 per cent hemolysis, — indicates > 50 per cent hemolysis.

fixation is due to an antigen-antibody reaction, the pronounced prozone indicates the presence of an antigen excess in low dilutions of the serum sample. This was assumed to provide a basis for overcoming these reactions. As they rarely took place with serum/antiserum concentration ratios exceeding 1:2 it was believed that the admixture of an equal amount of negative serum to the HCG-antiserum might extinguish these reactions because of the introduction into the system of a state of antigen excess to the point of complete inhibition of this antigen-antibody reaction. To test this hypothesis a series of two-dimensional titrations of an HCG-antiserum with a negative serum was performed. The antiserum had been premixed with various amounts of negative serum (from another patient), the serum/antiserum ratio varying from 1:8 to 1:1. A considerable reduction of the complement-fixing reaction was here observed already in the ratio 1:8 and the reactions were completely overcome with a serum/antiserum ratio of 1:4 (Table I). Titrations of antisera saturated with an equal amount of negative serum have never given positive reactions with negative sera.

The admixture of negative serum to HCG-antiserum may be carried out immediately before use of the antiserum. No decrease in the effect on the elimination of complement fixing reactions with negative sera has been noticed in saturated antisera stored for 3 to 4 weeks or more. It should here be mentioned that this saturation procedure hos no influence on the specific reaction between HCG and antiserum. No change in antibody titer was observed, nor was there any decrease in the sensitivity of the procedure in terms of maximal antigen dilution bringing about complete inhibition of hemolysis.

*Reactions between HCG-antisera and specimens from pregnant women.* Two-dimensional titrations of HCG-antisera with positive sera showed a rather uniform pattern. A typical example is illustrated in Fig. 2. The dependence of antiserum titer upon the HCG-concentration is clearly demonstrated. Comparatively low antiserum titers are found in the area of antigen excess[1]. A rather distinct zone of optimal antigen dilution is observed. In th

[1] Antigen excess: dilutions less than optimal antigen dilution.

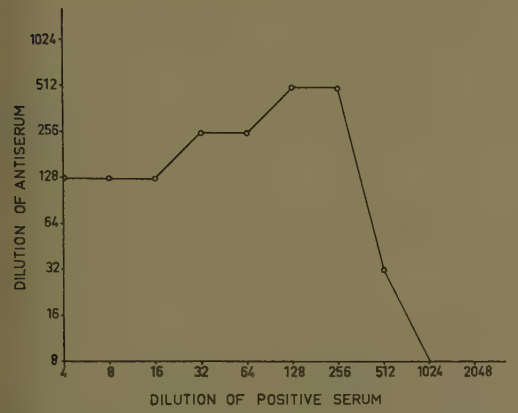



Fig. 2. Two-dimensional titration of HCG-antiserum with serum from a pregnant woman. The greatest antiserum dilution inhibiting hemolysis is indicated in the figure.

zone of antigen deficit[1] there is a rather abrupt decrease in antiserum titers. The curve, therefore, is not symmetrical.

*Heterologous reactions with HCG-antisera.* Fig. 3 shows a two-dimensional titration of HCG-antiserum with a human menopausal gonadotrophin (HMG) preparation[2]. It was dissolved in equal amounts of veronal buffer and negative serum, its concentration being 1.46 mg per ml. It is seen that the HCG-antiserum possesses the ability to react by complement fixation with HMG, this cross-reaction, however, only taking place with very high concentrations of HMG. In contrast to the conditions seen in titrations with the homologous antigen (HCG) the decrase in HMG-concentration is accompanied by a rather slow decrease in antiserum titers.

Pregnant mare serum gonadotrophin (Gestyl, N.V. Organon, Holland) was also tested with HCG-antiserum, but no positive reactions occurred with this hormone in a concentration of 400 I.U. per ml or less.

*Standardization of HCG-antisera for routine analysis of HCG.* The incorporation of an HCG-antiserum into a routine procedure for the quantitative determination of HCG necessitates the selection of an appropriate antiserum dilution, as such a procedure is preferably carried out as a one-dimensional tiration with serial dilution of the test sample only. For standardization of an antiserum a two-dimensional titration with a negative serum is performed. If complement is fixed, the antiserum is saturated with an equal volume of negative serum and retested to check the effect. Thereafter a two-dimensional titration is carried out with 1,500 I.U. of HCG dissolved in 1 ml of negative serum. A suitable antiserum dilution for routine analysis is chosen (Table II). We have preferred to use 4 to 8 antiserum units. One antiserum

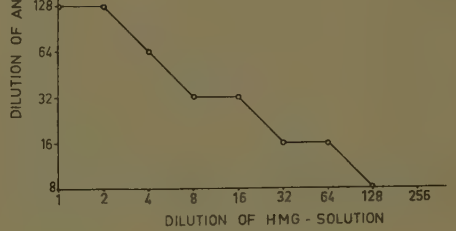



Fig. 3. Two-dimensional titration of HCG-antiserum with a HMG-preparation. The greatest antiserum dilution inhibiting hemolysis is indicated in the figure.

[1] Antigen deficit: dilutions more than optimal antigen dilution.

[2] The HMG-preparation was kindly provided by Dr. E. Diczfalusy. Its potency, as determined with the mouse uterine test, exceeds that of a reference preparation (HMG-20A) 22.5 times.

Table II. *Two-dimensional titration of HCG-antiserum and HCG.*

| Antigen | Dilution of antigen | Dilution of antiserum | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| HCG dissolved in veronal buffer, 1.500 I.U. per ml | 50 | + | + | + | + | − | − | − |
| | 100 | + | + | + | + | + | − | − |
| | 200 | + | + | + | + | + | + | − |
| | 400 | + | + | + | + | + | + | − |
| | 800 | + | + | + | + | + | + | − |
| | 1.600 | + | + | + | + | + | + | − |
| | 3.200 | − | + | + | + | + | + | − |
| | 6.400 | − | − | + | + | − | − | − |
| | 12.800 | − | − | − | − | − | − | − |

+ indicates no hemolysis.
— indicates complete hemolysis.
In the narrow region between complete and no hemolysis + is for simplicity used to indicate ≦ 50 per cent hemolysis, — indicates > 50 per cent hemolysis.

unit is here defined as the highest antiserum dilution completely inhibiting hemolysis at the optimal antigen dilution.

*Quantitative determination of HCG in serum.* The sample to be examined for the presence of HCG is serially diluted with veronal buffer, the initial dilution being 1:2. It is tested with antiserum standardized as described above. Simultaneously an HCG-standard of reference is tested. As standards of reference for serum specimens we have used either a positive serum, or HCG dissolved in a negative serum. Heating of the HCG-solutions at 56° C for 30 to 60 minutes had no deteriorating effect on the HCG-content, nor was it affected by storage at —20° C for more than 6 months or by repeated freezing and thawing. Occasional instability of HCG in veronal buffer, particularly in high dilutions, was, however, observed. No deterioration of the antisera was noticed in connection with repeated freezing and thawing.

The concentration of the hormone at the final antigen end-point indicates the sensitivity of this procedure. With the different antisera prepared according to scheme 4 it was possible to determine 0.10 to 0.50 I.U. of the hormone per ml.

*Accuracy of the serological procedure for the determination of HCG.* In order to evaluate the accuracy of the above-outlined procedure, 10 serum samples from pregnant women were run in duplicates on 10 different occasions. As these determinations were carried out at rather long intervals, several different batches of sheep blood were employed, whereas complement, amboceptor and HCG-antiserum were from the same pools. The serum samples were titrated in 1.25-fold serial dilutions and the HCG-antiserum was employed in the dilution 1:32.

Table III is a summary of the statistical calculations. As is seen, the variance for duplicate determinations ('within days') is 0.255. This implies that the standard deviation (s) is ± 0.505 dilution step. As the scatter in a mean value of n determinations is $s/\sqrt{n}$, it will in our case, where duplicate determinations were run, amount to ± 0.353 dilution-step. The variance for 'between days' ($s_d^2$), i. e. for determinations of the

Table III. *Survey of statistical data.*

| Source of Variation | Degrees of Freedom | Sum of Squares | Variance |
|---|---|---|---|
| Between sera | 9 | 37.05 | |
| Between days | 90 | 143.45 | 1.594 |
| Within days | 100 | 25.50 | 0.255 |
| Total | 199 | 206.00 | 1.035 |

ame serum sample carried out on different lays, is 1.594. The true standard deviation $(s_t)$ for 'between days' is calculated with ompensation for the scatter for 'within days' ccording to the formula

$$s_t = \sqrt{\frac{1}{n}(s_d^2 - s^2)}.$$

t is here ± 0.819 dilution-step.

The quantitative determination of HCG n a sample of unknown concentration of he hormone is carried out on the basis of imultaneous titrations of a standard of refer- nce. The 95 % confidence limits for an ob- ained mean value of a duplicate analysis is letermined by the formula antilog

$$g\left(\frac{R}{S}\times C\right) \pm 1.96 \times s \times \log(\text{dilution factor})\Big],$$

where R = mean value of end-point dilution f the standard of reference, S = mean alue of end-point dilution of the unknown ample, C = amount of HCG in I.U. per nl of the standard of reference, and s = tandard deviation for 'within days'. This mplies that the 95 % confidence limits, ex- ressed as a percentage of the obtained mean alue of duplicate determinations, are —20 % nd + 25 %.

*Clinical application of the serological pro- edure for determination of HCG.* The above- utlined procedure has been adopted for the letermination of HCG in more than 300 erum samples from 248 pregnant and 6 non-pregnant women, including wom- n after the menopause. In two instances pecimens were obtained in the first week fter a missing period. The other samples vere taken from patients throughout preg- ancy. Some of these results have been pub- ished earlier (Brody & Carlström 1961). The highest HCG-concentrations were found during the first 3 months of pregnancy and a rather slow decrease was noticed in later stages.

The diagnosis of pregnancy was confirmed by gynecological examination and in early cases by biological pregnancy tests[1]. All early cases were also checked later by clinical follow-up examinations. No false positive reactions were encountered. In 8 cases anti-complementary effects were observed, but could be overcome by treatment according to Taran (1946). In one instance a false negative reaction was observed. This specimen had on previous examination shown positive reactions, which was also the case on retesting. Technical error is the probable explanation.

## DISCUSSION

It was early recognized that prolonged parenteral administration to rodents of gonadotrophic hormone from human pregnancy urine gradually diminished the physiological effects of the hormone (Zondek 1931, Selye, Collip & Thomson 1934). This was believed to be due to the formation of an antigonadotrophic factor (Selye, Bachman, Thomson & Collip 1934, Bachman, Collip & Selye 1934). The presence of this factor was demonstrable only by its capacity to neutralize the hormonal activity *in vivo* (Selye, Bachman, Thomson & Collip 1934, Bachman, Collip & Selye 1934, Twombly 1936, Sulman 1937, Zondek & Sulman 1937 a-e, Freud & Uyldert 1947). Detailed studies on this antigonadotrophic factor were carried out by Sulman (1937) and Zondek & Sul-

[1] The biological pregnancy tests were performed at the Hormone Laboratory of the Department of Obstetrics and Gynecology, Karolinska sjukhuset.

man (1937a-e), who investigated its origin and preparation, specificity and mechanism of action.

Two requirements have to be fulfilled by an *in vitro* serological method for the determination of HCG in clinical conditions such as *e. g.* the diagnosis of pregnancy. One requirement refers to the specificity, *i. e.* the capacity of the antiserum to react with HCG in body-fluids without interference from other substances. The other desideratum concerns the sensitivity of the method in terms of the HCG-concentration at the final antigen end-point.

As regards the specificity it is evident that attempts to prepare HCG-specific hyperimmune sera have been unsuccessful until recently (Brody & Carlström 1960). Previously, precipitating and complement-fixing antibodies had been obtained by immunization of rabbits with gonadotrophic hormone prepared from human pregnancy urine (Bachman 1935, Twombly 1936, van den Ende 1939, Got, Levy & Bourrillon 1959). Considerably impure hormone preparations have, however, been used for the delevelopment of the antisera in all these studies as evidenced by the presence of uniform cross-reactions of the hyperimmune sera with the gonadotrophic preparation as well as with extracts from male urine (Bachman 1935), by the finding of equally strong reactions with normal male and female sera as with pregnancy sera (Bachman 1935), by the results of absorption experiments (van den Ende 1939), and by the effect on the immunoelectrophoretic pattern of purification of the antigen used for preparing the hyperimmune sera (Got, Levy & Bourrillon 1959). The presence of a large number of unspecific antibodies in the hyperimmune sera as prepared by these authors will therefore render impossible their incorporation in methods of sufficient specificity or sensitivity for the determination of HCG in body-fluids.

The purity of the antigen thus plays a decisive role in the attainment of the specificity of the antiserum. It is highly probable that a decrease in the antigenicity of a hormone preparation will take place as a result of the process of purification. Mixture of the antigen with an adjuvant will, however, considerably increase the antibody-inducing capacity of a substance with relatively low antigenicity. The HCG-preparation used in this study is claimed to be a highly purified compound containing 6,000 I.U. of the hormone per mg. The reported molecular weight of HCG is 100,000 (Gurin, Bachman & Wilson 1940), and the incorporation of this substance into an adjuvant should provide suitable conditions for antibody-formation.

As has been shown above, some HCG-antisera reacted in the complement-fixation test with negative sera. These reactions are similar to those observed by Witebsky, Rose & Shulman (1955) between hog thyroid antiserum and hog serum. These authors interpreted their reactions as being due to species-specific antibodies. The reactions observed in our system might conceivably be due to such species-specific antibodies but it cannot be excluded that they may be caused by antigenically active impurities of the antigen used for the immunization or by cross-reactions with substances antigenically related to HCG.

In an attempt to elucidate the homogeneity of the HCG-preparation used here and in order to shed further light on the nature of

the complement-fixing reactions with negative sera we have performed some gel-diffusion precipitation experiments. The three basin plate technique of Ouchterlony (1949) was used. In a previous paper we have reported negative results of such experiments (Brody & Carlström 1960). These were then performed with low-titer HCG-antisera obtained with immunization scheme 3. We have now repeated these experiments with a high-titer HCG-antiserum prepared according to scheme 4. In preliminary tests we have found three precipitation lines between the HCG-preparation and the HCG-antiserum. Two of these lines merge with the precipitate formed with undiluted, positive sera. Negative sera, diluted 1 : 5, showed a line which interfered with one of those formed between the HCG-preparation and the HCG-antiserum. This line could be eliminated with the saturation procedure.

These findings indicate that the HCG-preparation might not be antigenically homogeneous. Furthermore, it is of considerable interest to note that negative sera showed a reaction of partial identity in the comparative analysis with the HCG-preparation in the Ouchterlony test. These results taken together with the demonstration of the complete elimination of the complement-fixing reactions with negative sera by the simple absorption procedure outlined above, seem to indicate that reactions between negative sera and HCG-antisera are probably due to antigenically reactive impurities of the HCG-preparation. These studies will be reported in detail elsewhere.

It is of interest to find out whether cross-reactions occur between the HCG-antisera and gonadotrophic hormones other than HCG. We have therefore tested HMG and pregnant mare serum gonadotrophin. Other gonadotrophic preparations have so far not been available to us. Cross-reactions in the complement-fixation test occurred with high concentrations of HMG. The concentration of this hormone-preparation at the final antigen end-point was approximately 0.1 mg per ml at antiserum dilutions of 1:32 (Fig. 3), corresponding to about 2 mg per ml of a reference preparation (HMG-20A). As the routine HCG-titrations are begun in a dilution of the serum specimens of 1:2, this will necessitate HMG-concentrations in the specimen equivalent to 4 mg of HMG-20A per ml before any reaction occurs. This is a concentration far above those reported for physiological as well as some pathological states in women (Apostolakis & Loraine 1960). From an immunological point of view, however, the observed cross-reaction is of interest on account of the implications of a possible antigenic relationship between HCG and pituitary gonadotrophins. This problem is under study.

The sensitivity of the above-outlined serological procedure for the determination of HCG may be expressed by the concentration of HCG at the final antigen end-point. With HCG-antisera obtained with immunization scheme 4 we are able to determine concentrations of 0.10 to 0.50 I.U. of HCG per ml. This is a satisfactory sensitivity, exceeding those obtained by biological determination procedures (Hamburger 1950, Albert & Kelly 1958, Albert & Derner 1960).

We have for some time used the complement-fixation test in a routine procedure for the determination of HCG in serum. Some of these results have been

published earlier (Brody & Carlström 1961). In pregnant women positive findings were noticed already in the first week after a missing period. The highest HCG-concentrations were observed in the first trimester with a slow decrease during the following stages of pregnancy. The conventional HCG-concentration curve during pregnancy found with biological methods shows a distinct peak of HCG-concentration around 60 days after the last menstrual period and is followed by a relatively abrupt decrease in concentration (Browne & Venning 1936, Evans, Kohls & Wonder 1937, Boycott & Rowlands 1938, Rakoff 1939, Wilson, Albert & Randall 1949, Loraine 1950).

Since the presentation of our first report (Brody & Carlström 1960) there have been several publications which, in principle, confirm our results. McKean (1960) found precipitating antibodies against HCG, but has only applied this method to the qualitative estimation of HCG in urine. A method for the serological estimation of HCG in urine has also been proposed by Wide & Gemzell (1960), who employ a technique similar to that used by Read & Stone (1958) for the determination of growth hormone. Rao & Shahani (1961), finally, have investigated properties of HCG-antisera with serological *in vitro* and *in vivo* methods simultaneously.

## SUMMARY

The capacity of human chorionic gonadotrophin to induce the formation of complement-fixing antibodies has been investigated. A detailed report is given on the application of the complement-fixation test to the determination of HCG in serum. The specificity, sensitivity and accuracy of the method have been analyzed. Some results of its clinical use are given.

## ACKNOWLEDGEMENTS

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# CAPILLARY INDICATOR IN THE DETERMINATION OF CLOT LYSIS TIME

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Use of the lysis time of a clot composed of whole blood, plasma, fibrinogen *etc.* for determination of thrombolytic or fibrinolytic activity requires careful objective observation of the end-point of lysis. To establish it, use has been made of the tilting method, the bubbles arising from the clot (Permin, 1950; Lassen, 1958), the turbidity changes of the clot (Müllertz, 1952), sinking of sand (Marbet, 1958), a metal disk (Winterstein & Studer, 1955), or a glass bead (Fletcher, 1954) through the clot, and sedimentation of the fragments and red cells freed from the dissolved clot (Fearnley, Balmforth & Fearnley, 1957; Lackner & Goosen, 1959). The bubble method has been found particularly simple and accurate in several determination systems.

The authors, however, found it very difficult, often impossible, in studying lipemic plasma to observe the bubbles owing to the turbidity of the clot. Inaccuracy often arose in the sand method when the grains stuck together or remained in the meniscus. On the other hand, slow coagulation can complicate the determination even in clear plasma when an insufficient number of bubbles remain in the clot.

When the spontaneous or kinase-induced lysis of a clot composed of whole blood is rapid, determination based on the sedimentation of red cells and fragments is inaccurate since complete lysis may have taken place before sedimentation was observed (Lackner & Goosen, 1959). Nor did the tilting method give sharp end-points.

For these and other reasons the authors began to use a capillary to record the changing of a gel-like clot to fluid at the end-point of lysis (Permin, 1950 ;Dalby & Metz, 1957; Lassen, 1958; Marbet, 1958). A thin-walled glass capillary was immersed in the middle of the clot and the rising of the lytic product into the capillary showed the end-point of lysis. In order to facilitate observation, the capillary was treated in a special way.

## METHOD

*Preparation of the capillaries.*

Capillaries were drawn from a Na-Ca glass tube Ø 7 mm so thin that water rose in them by capillary force over 40 mm but less than 80 mm. The capillary was broken into parts of equal length. The authors generally employed a length of 120 mm.

The capillaries were dyed with tetrabromo-phenolsulphone-phthalein. The staining solution used was saturated alcohol solution of bromo-phenol blue[1] which contained about 8 per cent of 1/6 N HCl solution. The staining solution was drawn into the capillary by capillary force, leaving about 1 cm of the tube unstained. The capillaries were emptied preliminarily by means of filter paper and finally by centrifugation so that the staining solution stil

[1] Edward Gurr, Ltd.

in the capillaries was absorbed into the layer of absorbent cotton at the bottom of the centrifuge tube. It is possible with some experience to treat some thousands of capillaries in a few hours.

The finished capillaries were kept in sterile, tightly closed tubes in which they retained their orange color well.

*Use of the capillary.*

The method of use is similar in principle whatever the nature of the clot. After the clot has formed the capillary is thrust half way into the clot, unstained end first. Care must be taken to ensure that the tip of the capillary does not touch the tube wall. This method obviates the inaccuracy caused by the irregular lysis of the edges of the clot. Surface activation (Niewiarowski & Prou-Wartelle, 1959), retraction of the clot (Permin, 1950), and other factors can cause the formation of liquid between the tube wall and the clot. When this happens, the premature rise of the peripheral bubbles and slow absorption of the fluid into the capillary can be observed if it is touching the wall. If the capillary is placed correctly it drops to the bottom when the clot dissolves, and the liquid rises almost at once into the capillary. The faintly orange color of the capillary now turns an intense blue-violet, which makes it easy to record the rise. Lysis is regarded as having occurred when the liquid has risen in the capillary to a certain height from the clot surface.

For determination of the proactivator activity with standard cattle plasma clot, 3—100 per cent cattle plasma was used. The capillary functioned faultlessly and with roughly the same accuracy in the different dilutions. The deviation of single determinations was tested in an experiment with 10 per cent cattle plasma. Twenty determinations gave a mean time of 228.4 sec.; range 225—231 sec.; S. D. = 1.27, *i.e.* 0.56 per cent.

For the lysis time of a clot composed of streptokinase-activated human plasma or whole blood, the deviation of single determinations was of the same magnitude as in the proactivator determination. It was possible with the capillary to determine the lysis of streptokinase-activated whole blood clot accurately even when the lysis times were under 2 min. The same was true with streptokinase-activated euglobulin the lysis times of which are short and in which frequent tilting of the tube interferes with normal lysis (Permin, 1950; Müllertz, 1952).

## DISCUSSION

Used in different clots and over 10,000 determinations in which the lysis times ranged from one to 35 min. the capillary proved to be a reliable indicator of the end-point of lysis. Even in the relatively few cases in which the lysis times were longer the capillary functioned. The determinations were made at 20°—37° C.

The authors compared the capillary with other indicators, *e. g.* quartz sand Ø 0.1 mm, quartz grits Ø about 1 mm, glass beads Ø about 4 mm, and the bubbles arising from the clot. All of them gave slightly differing but comparable results in normal conditions.

The capillary was developed principally for lipemic and whole blood clot. There was no inaccuracy caused by disturbing factors such as variation in clot consistency, boundary surface adsorption, evaporation, sticking together of the grains of sand and adsorption of streptokinase onto the tube wall (Lassen, 1958) when the capillary was used.

The first capillaries used by the authors were stained all over. A blue-stained zone was observed in the immediate vicinity of these capillaries which dissolved slightly more slowly than the clot. This disturbance, probably caused by bromophenol blue, did not occur when only the inner surface was stained.

## SUMMARY

By immersing glass capillaries treated with bromophenol blue into the middle of the clot, it was possible to determine accurately the lysis time of the clot in conditions in which other indicators failed.

Independently of the nature of the clot the capillary is serviceable over a relative wide time — and temperature range.

It is moreover possible to eliminate a number of sources of error by placing the capillary inside the clot.

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# DEVICE FOR SCANNING TRANSPARENT ELECTROPHORESIS STRIPS

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Small-scale electrophoresis of serum proteins was made according to the technique of Kohn (1958). The cellulose acetate strips were stained with Lissamine Green S.F.150 (Imperial Chemical Industries Ltd.) 0.3 per cent w/v in 15 per cent v/v acetic acid for 30 min. and washed in 2 per cent v/v acetic acid. The strips were made transparent by immersion in paraffine oil, as described by Consden & Kohn (1959). The transparent strips were mounted between two 25 × 75 mm microscopic slides, care being taken to press out trapped air. The mounted strips were fastened in the holder of the cuvette carrier of a Beckman model B spectrophotometer using plasticine or a metal support. The albumin was orientated toward the front part of the instrument.

For the scanning procedure a millimeter scale (0—50 mm) was fastened on the front panel of the instrument and a pointer was attached to the knob which is used for moving the cuvette holder, as seen in Fig. 1. The parts are easily removed when the instrument is to be used for ordinary purposes. The scale A in Fig. 1 consists of a millimeter paper wrapped around a metal support. The scale can be moved along the support if the bolt B with its nut is loosened, since the metal support is provided with a slit for the bolt. Before the dry electrophoresis strip is immersed in paraffine oil, a mark is made on it with a pencil in front of the albumin fraction. The pencil mark and the zero position of the millimeter scale should correspond. The pointer needle C rests on the millimeter

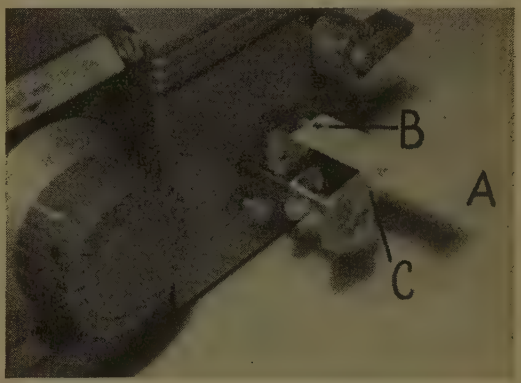


Fig. 1.

scale. By adjusting the screw at C the cuvette holder is made to run smoothly.

The millimeter scale is brought into zero position by moving the cuvette holder with the mounted electrophoresis strip in the light beam with the shutter open, until the galvanometer indicates the pencil mark. The bolt B is then fastened and the blank value is read at 620 mμ on an unstained part of the strip. The slit value has been 0.22—0.23 mm. The strip blank value is checked a few times during measurement. The readings are made at intervals of 0.5 or 1.0 mm. About 60 readings have been made for every strip. The readings

are plotted on graph paper. The protein fractions are separated perpendicularly, cut and weighed on an analytical balance. The amount of protein in the fractions is calculated from the known concentration of total protein in serum. By incorporation of an integrating device and a strip chart recorder, much time could be saved. This has, however, not been done by the present author.

The following normal values for the principal serum protein fractions were obtained using the described technique (20 blood donors, 8 women and 12 men, aged between 18 and 57 years): albumin 4.86 g/100 ml (range 4.35—5.24), alpha$_1$-globulins 0.22 g/100 ml (range 0.17—0.35), alpha$_2$-globulins 0.49 g/100 ml (range 0.27—0.60), beta-globulins 0.67 g/100 ml (range 0.49—0.85), and gamma-globulins 0.99 g/100 ml (range 0.74—1.41).

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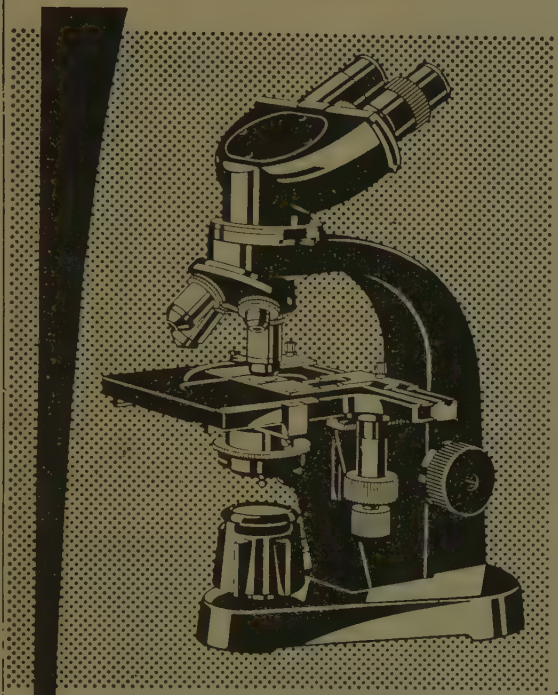

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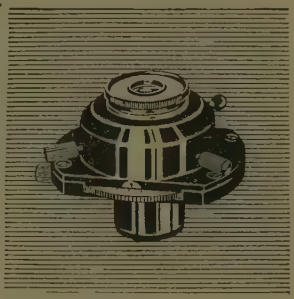

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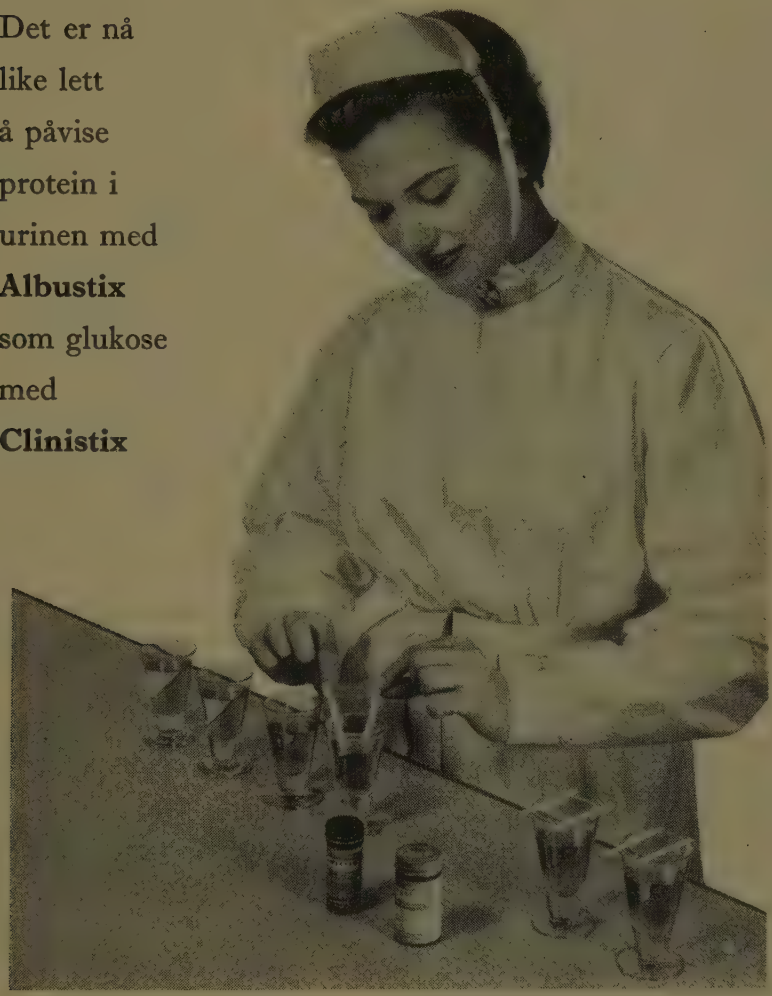

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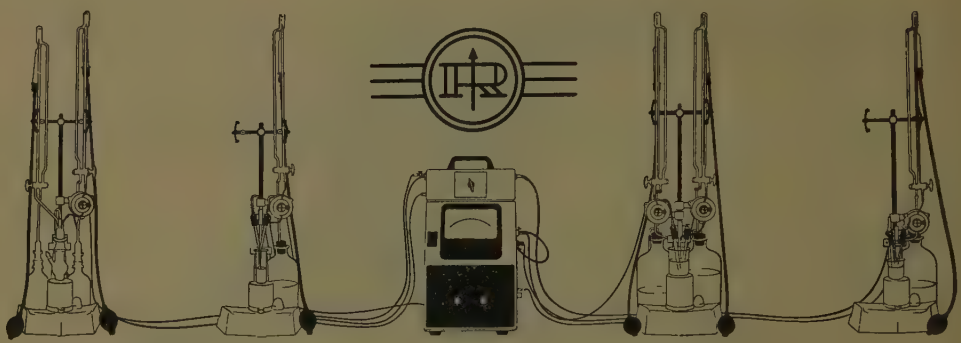

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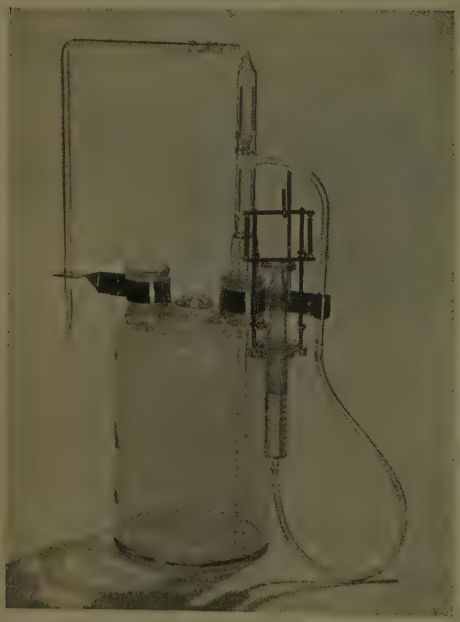

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